UNIVERSITÀ DI PISA

FACOLTÀ DI SCIENZE MATEMATICHE FISICHE E NATURALI

CORSO DI LAUREA MAGISTRALE IN BIOLOGIA APPLICATA ALLA

BIOMEDICINA

CURRICULUM NEUROBIOLOGICO

ANNO ACCADEMICO 2014-2015

APPELLO DI LAUREA – OTTOBRE 2015

CANDIDATO - ALEXIA TIBERI

TESI DI LAUREA MAGISTRALE

CHARACTERIZATION OF MICROGLIA DURING NGF DEPRIVATION IN VITRO AND IN A MOUSE MODEL OF ALZHEIMER'S DISEASE

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INTRODUCTION

1. ALZHEIMER'S DISEASE

In 1906, during a psychiatric meeting of the South West German Society of Alienists, *Dr. Alois Alzheimer* presented the case of Auguste D., a 51-year-old woman who had been admitted to a hospital in 1901 with an unusual cluster of symptoms that included reduced comprehension and memory, aphasia, disorientation, unpredictable behavior, paranoia, auditory hallucinations, and pronounced psychosocial impairment. She died on April 8, 1906. Alzheimer published this case report with the title "*On an Unusual Illness of the Cerebral Cortex*"(Alzheimer, Stelzmann et al. 1995). Since then much progress has been made in discovering the molecular and genetic underpinnings underlying that particular disease, shedding light in what is still one of the most enigmatic subjects in medicine – Alzheimer's disease.

Alzheimer's disease (AD) is the eponym of neurodegenerative diseases. It is the most common cause of dementia affecting 44.4 million people today, a number expected to reach 135.5 million worldwide by 2050 due to the ever increasing average age of the population (http://www.alzheimers.net/ resources /alzheimers-statistics/).

Given both social and economic burden that such a devastating disease has on today's world, it is not surprising the enormous effort that the scientific world is putting into finding a definitive treatment. But we are still very far from reaching that goal and getting there means acquiring more knowledge on the pathogenesis of the disease and elucidating its biology.

1.1. HISTOPATHOLOGICAL HALLMARKS

The histological lesions that Dr. Alzheimer himself observed – and that are still the first and foremost diagnostic feature – are amyloid plaques and neurofibrillary tangles, and it is the presence of these aggregates that puts AD into the category of the "protein misfolding diseases" (Brettschneider, Del Tredici et al. 2015).

Amyloid (or senile) plaques are extracellular, insoluble aggregates of amyloid β-peptide (Aβ) (Glenner, Wong et al. 1984). Neurofibrillary tangles, on the other hand, (NFTs) are intracellular cytoplasmic bundles of paired, helically bound 10 nm filaments (PHFs). NFTs are polymers of tau, a microtubule-associated protein (MAP) that has the

physiological role of stabilizing axonal microtubules and it is usually soluble. However, when hyperphosphorylated, tau becomes insoluble and gains the ability to self-assemble into tangles (Grundke-Iqbal, Iqbal et al. 1986).

Neuroanatomically, these lesions show two completely different localizations and spreading directions. Aβ plaques appear first in the cortex (particularly the prefrontal cortex-PFC) and then propagate in more posterior regions toward the hippocampus, the enthorinal cortex(EC) and the nucleus basalis of Meynert (Braak and Braak 1991). NFTs, on the other hand, follow the opposite route, starting from the hippocampus and EC and only later spread to the cortex (Musiek and Holtzman 2015).
This unusual propagating inclination of the disease's features, where a sort of "contagion" between adjacent regions seems to suggest itself, has been time and again labeled a "prion-like" self-propagating mechanism, implying that misfolded proteins associated with AD are able to function as a template to induce misfolding in "healthy" proteins and as a seeding point for the formation of new aggregates (Brettschneider, Del Tredici et al. 2015)..

These two classical lesions can actually occur independently in humans. Aggregates of tau that are very similar to those seen in AD have also been described in more than a dozen neurodegenerative diseases in which one generally finds no Aβ deposits and neuritic plaques. Conversely, Aβ deposits are seen in aged "normal" cortex in the virtual absence of tangles. Moreover some cases of AD are said to be "tangle-poor" meaning very few NFTs in the neocortex (Terry, Hansen et al. 1987). In this peculiar cases other types of inclusions are found, the Lewy body, composed principally of fibrils of α-synuclein which is actually an histological sign of Parkinson's disease(Hansen, Masliah et al. 1993). NFTs, independently of Aβ plaques, can thus arise in the course of a variety of etiologically different neuronal insults (Selkoe 2011). Interestingly, tau pathology correlates much more closely to neuronal loss, both spatially and temporally, than amyloid plaques do (Musiek and Holtzman 2015).

These microscopic lesions have provided the starting point for approaching the molecular pathogenesis of AD but, given the cytological and biochemical complexity of the disorder, it has been difficult to come to an agreement about the temporal sequence of events that lead to dementia; and even though there are still many open questions,

much evidence implicates that Aβ and tau species make an important contribution to disease progression.

1.2. GENETIC AND MOLECULAR BASIS OF AD

AD is considered genetically a dichotomous disease existing in two forms: early-onset "familial" AD (EOAD) and the more frequent late-onset "sporadic" AD (LOAD) (Tanzi 2012). Linkage studies identified mutations in three genes as the primary cause of familial AD: amyloid precursor protein (APP), presenilin 1 (PSEN1), and presenilin 2 (PSEN2) (Rao, Degnan et al. 2014). EOAD is characterized by Mendelian inheritance so the genetic factor is the prevalent one in the pathogenesis of the disease.

Speaking of APP, despite the extensive research driven by its association with AD, still the evidence of potential biological roles for the amyloid precursor is circumstantial and a defined function remains elusive.

APP belongs to a family of evolutionarily and structurally related proteins, thus a number of homologous sequences have been identified either in mammalian and non mammalian organisms(Muller and Zheng 2012). In humans, the APP gene is located on chromosome 21, contains 18 exons and extends over 240 kilobases (Yoshikai, Sasaki et al. 1990).

APP can undergo either one of two metabolic processes: the amyloidogenic and the anti-amyloidogenic pathway. The protein can be cleaved by one of the α-secretases, which are members of the ADAM (A disintegrin and Metalloproteinase) family of membrane-anchored metalloproteases responsible for degrading many components of the extra cellular matrix, releasing the soluble ectodomain region (APPs-a) and leaving behind a membrane-retained C-terminal fragment (CTF) of 83 amino acids (C83). The C83 is then cut by the γ -secretase to generate a peptide (p3)(Esch, Keim et al. 1990, Sisodia, Koo et al. 1990, Haass, Hung et al. 1993). This is referred to as the anti-amyloidogenic pathway.

Alternatively, APP is processed by the β-secretase, releasing a truncated form of APPs (APPs-b) from the cell and leaving a 99-residue CTF (C99) in the membrane. The β-secretase, called β-site APP cleaving enzyme 1 or BACE1, is a single-transmembrane aspartyl protease with its active site in its ectodomain. C99 is subsequently cleaved by the γ-secretase releasing Aβ into vesicle lumens and extracellular fluid (Seubert, Oltersdorf et al. 1993). Interestingly, PSEN1 and PSEN2 are part of the γ-secretase

complex thus adding another link between Aβ production and AD (Gu, Misonou et al. 2001, Sastre, Steiner et al. 2001, De Strooper 2003).

No evidence has emerged that a fundamental function of APP is lost in AD patients. Instead, AD-causing APP mutations are all clustered near the secretase processing sites and seem to act by a gain-of-toxic-function mechanism, namely the increased production of the Aβ fragment, particularly Aβ42(Karch, Cruchaga et al. 2014). The same increase in the production of Aβ happens in Down syndrome patients(this is definitely no surprise since they have a trisomy of chromosome 21) and therefore develop Aβ plaques themselves(Karch, Cruchaga et al. 2014).
Interestingly enough, mutation in APP have been detected also in sporadic cases of Alzheimer - LOAD. This type of AD is actually the most common one and is defined by a late age of onset (> 65 year). It is strongly influenced by both genetic and environmental factors thus making it a complex multifactorial disease with hard-to-pinpoint factors and causes underlying its pathological processes.

Genetic evidence strongly supports the idea that both familial and sporadic AD might actually be caused by overproduction and accumulation of Aβ. This idea is generally referred to as the "amyloid hypothesis" (Heppner, Ransohoff et al. 2015), an hypothesis that since its first conception has undergone profound conceptual evolution. In the most basic version of today's hypothesis, various forms of Aβ are created on the road from the monomer to the fibril, and these "oligomers" are believed to be the most toxic form of amyloid, leaving plaques to be simple reservoirs of these smaller, potentially neurotoxic assemblies (Heppner, Ransohoff et al. 2015).

Consistent with this idea, both synthetic Aβ oligomers and soluble Aβ oligomers isolated from cell culture media or AD brain extracts have been shown capable of impairing synaptic functions when added to hippocampal slices or slice cultures (Shankar, Li et al. 2008).

The link between pathology and physiology stands in the natural role of Aβ. Evidence shows that the production of Aβ and its secretion into the extracellular space depend on neuronal activity both in vitro (Kamenetz, Tomita et al. 2003) and in vivo (Cirrito, Yamada et al. 2005). Increased neuronal activity enhances Aβ generation and blocking

neuronal activity has the opposite effect (Kamenetz, Tomita et al. 2003). This synaptic regulation of Aβ production is mediated, at least in part, by clathrin-dependent endocytosis of surface APP at presynaptic terminals, endosomal proteolytic cleavage of APP, and Aβ release at synaptic terminals (Cirrito, Yamada et al. 2005). Continuous overproduction of Aβ at dendrites or axons has been seen to act locally to reduce the number and plasticity of synapses(Wei, Nguyen et al. 2010).

In this perspective, a negative feedback loop likely exists, where Aβ is produced in response to neuronal activity and increased levels of extracellular Aβ near synapses causes a reduction of excitatory transmission. In pathological conditions, high levels of Aβ, due to a mutation like in EOAD or a combination of both genetic and environmental factors like in LOAD, could disrupt the feedback loop, suppressing altogether excitatory synaptic activity at the postsynaptic level(Mucke and Selkoe 2012). Thus synaptic dysfunction starts before the appearance of plaques and is carried out by the oligomeric form of the Aβ peptide. This is in accordance with evidence in human patients, where MCI seems to correlate most with soluble aβ levels than with plaques(Musiek and Holtzman 2015). These interesting considerations have moved much of the effort of the scientific community into developing a treatment for AD that could decrease Aβ burden. Up until now, every clinical trial yielded at best shaky results. The possibility that Aβ initiates pathology very early in the disease suggests that only early anti-Aβ therapy is likely to be effective and , to date, no completed trial has attempted therapy early in the Aβ cascade (Musiek and Holtzman 2015).

Regarding LOAD, genetics bring additional useful information into the picture. Linkage studies identified on chromosome 19 an important risk factor, APOE. The apolipoprotein E is the major cholesterol carrier in the brain and is involved in neuronal maintenance and repair. APOE binds to several receptors on the cell surface, which are involved in lipid delivery and transport, glucose metabolism, neuronal signaling, and mitochondrial function. Normally, APOE binds to the Aβ peptide and plays a role in its clearance(Bu 2009).

APOE occurs as three possible isoforms that differ based on two amino acid residues (112 and 158): APOEε2, APOEε3, and APOEε4. APOEε3 is the most common APOE isoform, occurring in approximately 72% of the population (Guerreiro, Gustafson et al. 2012). The ε4 allele apparently increases risk of LOAD 3 fold in heterozygotes and 10 fold in the omozygotes and also anticipates the age of onset for either LOAD or EOAD.

On the other hand, APOEε2 decreases risk for late-onset AD and delays age at onset(Guerreiro, Gustafson et al. 2012, Karch, Cruchaga et al. 2014)

Thanks to new technologies such as GWAS and whole-exome sequencing/whole-genome sequencing strategies, many new genes have been uncovered shedding some light in the many pathogenic processes involved in LOAD(for a comprehensive review on the specific genes (Karch, Cruchaga et al. 2014)). Of notice, genes detected by these studies are involved in lipid metabolism(APOE, CLU, ABCA7, and SORL1), endocytosis (BIN1, PICALM, CD2AP, EPHA1, SORL1, RIN3, MEF2C, and MADD) and immune response(R1, CD33, MS4A, CLU, ABCA7, EPHA1, HLA-DRB5-DRB1, INPP5D and TREM2) (Karch, Cruchaga et al. 2014). These genetic factors do not necessarily affect APP proteolysis or increase Aβ production, and it is thus been suggested that it is a dysregulation of Aβ clearance the etiologic driving force in sporadic AD(Mawuenyega, Sigurdson et al. 2010).

Moreover the results pointed towards a whole new field in AD that lately has been flourishing: neuroinflammation (see chapter 1.4).

1.3. NGF AND ALZHEIMER

The discovery of the vulnerability and degeneration of the basal forebrain cholinergic neurons (BFCNs) in AD (Whitehouse, Price et al. 1982), has given ground to the so called "cholinergic hypothesis" (Bartus, Dean et al. 1982). Indeed, as producers of the neurotransmitter acetylcholine, BFCNs are involved in higher cognitive functions such as learning and memory and hypofunctionality in these neurons has been linked to cognitive deficits (reviewed in (Schliebs and Arendt 2006)).

The neurotrophin Nerve Growth Factor (NGF) (Levi-Montalcini 1952) is produced from a precursor (proNGF) which is cleaved, mainly by furin, and other metalloproteases, to mature NGF (Shooter 2001). Mature NGF is a known indispensable trophic factor for BFCNs. NGF has been shown not only to prevent death and promote synaptic cholinergic function in rodent and non-primates (Tuszynski 2007), but also to possess an anti-amyloidogenic activity in vitro and in vivo (Matrone, Ciotti et al. 2008, Capsoni, Brandi et al. 2011).

A disregulation of the NGF signalling can be detected in AD patients where, even though mRNA of the neurothrophin does not change (Jette, Cole et al. 1994), the protein levels increase in the cortex and in the hippocampus while they decrease in the basal forebrain (Fahnestock, Scott et al. 1996). Interestingly, TrkA expression shows a

marked decrease both in the BFCN and neocortex in AD brains and loss of TrkA expressing BFCNs has been described in mild cognitive impairment (MCI) and in early AD neuropathological stages (Counts et al., 2004; Ginsberg et al., 2006).

Remarkably, p75NTR receptor levels remain unchanged in AD (Ginsberg, Che et al. 2006), an unbalance in the TrkA/p75NTR ratio (and thus signaling) that might possibly cause dysfunction (Cattaneo, Capsoni et al. 2008).

Of notice, NGF is axonally retrotransported to the cell bodies of the BFCNs and this could be linked to the cytoskeletal transport dysfunctions seen in AD (Mufson, Conner et al. 1995). The very same APP has been shown to be capable of disrupting axonal transport (Gunawardena and Goldstein 2001). These data might indicate a negative neurodegenerative loop leading to reduced NGF reaching the BFCNs.

Another possible link between NGF and AD goes through proNGF. ProNGF is the most abundant form of NGF in the brain, and its levels show a marked increase in AD and MCI (Fahnestock, Michalski et al. 2001, Peng, Wuu et al. 2004). The pro form of the neurotrophin NGF can trigger cell death, as shown in cellular models using the purified protein (Lee, Kermani et al. 2001) or AD brain extracts (Pedraza, Podlesniy et al. 2005). In addition, evidence was provided linking NGF deprivation to the activation of Aβ aberrant processing of APP and tau in vitro (Tan, Town et al. 1999, Matrone, Ciotti et al. 2008, Matrone, Di Luzio et al. 2008) and in vivo, from the studies in the anti-NGF AD11 mouse model (see below).

1.3.1. THE AD11 MOUSE MODEL

The in vivo link between NFG deprivation and AD has been determined thanks to the mouse model AD11 that produces specific antibodies against the mature murine form of NGF, αD11(Cattaneo, Rapposelli et al. 1988, Paoletti, Covaceuszach et al. 2009).

αD11 is a monoclonal antibody, produced in rat, that has the ability of effectively neutralize NGF both in vivo(Molnar, Tongiorgi et al. 1998, Ruberti, Capsoni et al. 2000) and in vitro(Berardi, Cellerino et al. 1994, Molnar, Tongiorgi et al. 1998). AD11 mice actually produce a recombinant form of the antibody derived from the assembly of the variable regions with the constant regions of the k and h chains of human immunoglobulins. This facilitates the distinction of the transgenic antibody from the endogenous murine Igs. Both chains are under the control of the early region of the human CMV promoter which makes them ubiquitously expressed (Ruberti, Bradbury et al. 1993), were cloned in two separate plasmids and injected in different mouse oocytes.

To create a functional antibody both the heavy and the light chain must be expressed in the same cell and this is achieved by crossing VH and VK mice lines. Double marking assays confirmed the coexpression in a high number of neuronal and non neuronal cells. The level of coexpression increases with age (Ruberti, Capsoni et al. 2000).

If we look to the timing of the expression, antibody levels are not detectable between E15 and P45, but reached high levels at P90 (Capsoni, Ugolini et al. 2000). Postnatal expression is an important and favourable fact about AD11 mice because depriving the developing embryo of NGF provokes profound effects such as death at early postnatal ages (Crowley et al, Cell 1994) and thus prevents the analysis in an adult animal as it is expected to be a mouse model for AD.

The AD11 mouse shows loss of BFCNs and presents a form of neurodegeneration very similar to AD with amyloid plaques derived from the endogenous APP gene, hyperphosphorilated tau, NFTs, loss in synaptic plasticity and memory (see **TABLE 1** for a more comprehensive view on AD11 phenotype) (Capsoni, Ugolini et al. 2000, Capsoni, Giannotta et al. 2002, Origlia, Capsoni et al. 2006).

Going a little bit into detail regarding the temporal progression of neurodegeneration in AD11 mice, tau hyperphosphorylation and cellular redistribution of MAP2 were observed as early as 2 months of age at the level of the entorhinal cortex and hippocampus, and only at later stages reached other cortical regions. Neurodegeneration in the BFCN (detected by staining for choline acetyl-transferase) was observed from 2 months onwards. Extracellular deposits of Aβ were detected at 6 months of age and increased with age (Capsoni, Giannotta et al. 2002). Cerebrovascular Aβ deposition is found consistently throughout cerebral cortex, hippocampus, and thalamus of aged AD11 mice with antibodies against Aβ1–40, Aβ1–42 and Aβ17– 24 and is completely absent in nontransgenic mice. Activated microglia is present surrounding plaques and in the parenchyma (Capsoni, Giannotta et al. 2002). AD11 mice display an increased expression of presenilin 1 and β-secretase (BACE) (Capsoni, Giannotta et al. 2004).

AD11 mice present also evidence of synaptic failure that, in accordance with the amyloid hypothesis, should be the early driver of the disease. In the cortex, AD11 shows a progressive impairment of long term potentiation (LTP). At 2 months of age, LTP can be induced but not maintained but this failure can be reverted by acute administration of acetylcholine or of the cholinesterase inhibitor galantamine(Origlia,

Capsoni et al. 2006). In 9-10 months old AD11 mice, LTP can not be either induced or rescued by cholinergic treatment (Origlia, Capsoni et al. 2006).

Concerning the hippocampus, AD11 displays both short and long term plasticity deficits in the dentate gyrus (Houeland, Romani et al. 2010). Moreover, nicotine fails to modify synaptic strength and to enhance the frequency of spontaneous miniature excitatory postsynaptic currents in the CA1(but not in the CA3)(Sola, Capsoni et al. 2006) and this process seems to involve the inhibitory circuit(GABAergic interneurons)(Rosato-Siri, Cattaneo et al. 2006).

Indeed, in adult AD11 mice, GABAergic signaling shifts from a hyperpolarizing to an immature depolarizing state (Lagostena, Rosato-Siri et al. 2010), which might constitute a compensatory mechanism for the loss of excitatory input.

This picture fits with the excitatory imbalance proposed by the amyloid hypothesis and it is in accordance with what has been found in other mouse models of AD (Palop, Chin et al. 2007).

AD11 mice exhibit visual and spatial memory deficits that worsen over time. Transgenic mice start performing poorly at the ORT(object recognition test) at 4 months of age(De Rosa, Garcia et al. 2005) while deficits in spatial memory appear later (9 months of age), as assessed by using the MWM (Morris water maze) and OLT(object localization test). The late appearance of behavioral deficits fit well with the pathogenesis of AD in humans.

In summary, the AD11 mice display a comprehensive neurodegeneration that is reminiscent of the sporadic forms of AD.

But what about the so-called presymptomatic phase? What are the molecular processes happening in those first few months leading to the AD11 phenotype? Changes in global gene expression were assessed by microarray mRNA analysis plus RT-PCR validation, and surprisingly, interesting differences could be detected already at P30, when no overt pathology is present (D'Onofrio, Arisi et al. 2011). The most significant differentially regulated mRNAs cluster in three families: Wnt signaling, synaptic neurotransmission and inflammation/immune response. Quantitatively speaking the most differentially expressed mRNA are involved in inflammation and this is consistent with what is seen in genetic studies for AD (Karch, Cruchaga et al. 2014). Complement C3 mRNA is highly up-regulated at P30 in cortex (5.10 ratio) and hippocampus (5.16 ratio) and at P90 in all brain areas (9.67 and 11.62 ratio in cortex and BF, respectively), C1qa mRNA

is up-regulated in cortex and hippocampus at P90 and the C1qb is up-regulated in the BF and the hippocampus at P90, while C1qc expression is unchanged in the different brain areas and C2 mRNA is down-regulated in the BF and cortex at P30 and in the hippocampus at P90.

On this perspective the neurodegenerative phenotype in AD11 mice is more complex and intriguing compared to what one would on the basis of the simple direct NGF/cholinergic connection. Though the neurodegeneration must be due to the NGF deprivation, since delivery of NGF to the brain induces a complete reversion of the phenotype (Capsoni, Giannotta et al. 2002, De Rosa, Garcia et al. 2005). The specific mode of neutralization of NGF might be of notice since the Fc constant region of the antibody could entail additional effects as opposed to a full gene knock out approach. On the other hand the mere presence of an antibody in the brain is not sufficient to induce the neurodegeneration since other neuroantibody transgenic mice (Piccioli, Di Luzio et al. 1995) do not develop the same phenotype.

The remarkable, early activation of the complement cascade in the AD11 model is noteworthy, because of the recently demonstrated role of the complement system in mediating CNS synapse elimination, during normal brain developing (Stevens, Allen et al. 2007). In particular, this “synaptic pruning” is mediated by a particular cell type, the microglia (Paolicelli, Bolasco et al. 2011), pointing toward these brain immune cells as the possible mediators of the AD11 phenotype.

These last considerations bring us to the last part of this dissertation: the relationship between AD and microglia/neuroinflammation.

Table 1. Summary of Phenotypic Characterization in AD11 Mice

Phenotypic Markers	Brain Areas	Age (Months)								
		1	2	4	6	8	9	10	12	15
Detectable anti-NGF antibody in serum and brain		-	+	+	+	+	+	+	+	+
Shrinkage of superior cervical ganglia		ND	+	ND	ND	ND	ND	ND	ND	ND
Shrinkage of dorsal root ganglia		ND	+	ND	ND	ND	ND	ND	ND	ND
Decreased TrkA expression in trigeminal ganglia		ND	+	ND	ND	ND	ND	ND	ND	ND
Decreased expression of TrkB or TrkC in vestibular and nodose ganglia		ND	-	ND	ND	ND	ND	ND	ND	ND
Decreased sympathetic innervation in spleen		ND	+	ND	ND	ND	ND	ND	ND	ND
Apoptosis in the spleen		ND	+	ND	ND	ND	ND	ND	ND	ND
Decrease in number of IgG+ lymphocytes		ND	+	ND	ND	ND	ND	ND	ND	ND
Inflammatory response		++	++	ND	ND	ND	ND	ND	ND	ND
Choline acetyltransferase reduction	Basal forebrain	-	+	+	++	++	++	++	++	++
TrkA reduction	Basal forebrain	-	+	+	++	++	++	++	++	++
$P75^{NTR}$	Basal forebrain	-	-	+	++	++	++	++	++	++
Hyperphosphorylated tau*	Entorhinal cortex	-	+	+	++	++	++	++	+++	+++
	Occipital cortex	-	-	+	+	+	+	++	++	+++
	Parietal cortex	-	-	+	+	+	++	++	+++	+++
	Hippocampus	-	-	+	+	+	++	++	++	+++
Dystrophic neurites (tau-positive)	Cerebral cortex	-	-	-	-	+	+	++	++	+++
	Hippocampus	-	-	-	-	-	+	+	++	++
ALZ50-positive neurons	Cerebral cortex	-	-	-	-	-	-	+	++	++
Neurofibrillary tangles (NFT200 antibody)	Cerebral cortex	-	-	-	-	-	-	-	+	+++
Paired helical filament-1-positive neurons	Cerebral cortex	-	-	-	-	-	-	-	-	++
Insoluble tau (western blot)	Cerebral cortex	-	-	-	-	-	-	-	-	++
	Hippocampus	-	-	-	-	-	-	-	-	++
Paired helical filaments	Cerebral cortex	-	-	ND	ND	ND	ND	ND	ND	ND
Microtubule associated protein-2 altered distribution	Cerebral cortex	-	+	+	++	++	++	++	+++	+++
	Hippocampus	-	-	+	++	++	++	++	+++	++
Accumulation of Aβ	Hippocampus	-	-	-	+	+	++	++	+++	+++
Aβ plaques	Hippocampus	-	-	-	-	-	-	-	++	+++
Gallyas staining	Hippocampus	ND	-	-	ND	ND	ND	ND	ND	+
Bielschowsky staining	Hippocampus	ND	-	-	ND	ND	ND	ND	ND	+
Campbell-Switzer staining	Hippocampus	ND	-	+	ND	ND	ND	ND	ND	+
Neuronal cell loss	Cerebral cortex	-	-	-	-	+	+	+	++	++
DNA fragmentation	Cerebral cortex	-	-	-	-	-	-	-	-	+
Cortical long-term potentiation deficit	Cerebral cortex	-	+	ND	++	ND	+++	ND	ND	ND
Nicotine enhancement failure	Hippocampus	ND	ND	ND	+	ND	ND	ND	ND	ND
Abnormal GABA excitatory action	Hippocampus	ND	ND	ND	+	ND	ND	ND	ND	ND
Synaptic plasticity deficits in dentate gyrus	Hippocampus	ND	ND	ND	++	ND	ND	ND	ND	ND
Object recognition test deficit		+/-	+/-	+	+	++	++	+++	+++	+++
Object location deficit		ND	ND	ND	-	ND	ND	ND	ND	ND
Object context deficit		ND	ND	ND	+	ND	ND	ND	ND	ND
Radial maze (retention)		ND	-	+	-	++	ND	ND	ND	ND
Morris water maze deficit (acquisition)		ND	-	-	-	+	+	++	++	++
Morris water maze deficit (retention)		ND	-	-	-	-	-	++	++	++

*Revealed using mAb AT8.
ND, not determined.

TABLE 1: AD11 phenotypic features from(Capsoni, Brandi et al. 2011)

1.4. NEUROINFLAMMATION AND AD: MICROGLIA COME INTO PLAY

The past two decades of research into the pathological mechanism of AD have been largely dominated by the amyloid hypothesis: the neuroinflammation associated with AD was seen merely as a physiological response to pathological events. New data though, have recognized a more central role for the immune system as a true driving force in AD pathogenesis (Heppner, Ransohoff et al. 2015). However, the concept of neuroinflammation usually associated with diseases such as multiple sclerosis needs some refinement when applied to AD since it involves the innate immune system and a specific cell type, the microglia.

1.4.1. MICROGLIA

Microglia represent around 10% of the cells in the adult CNS though there is much heterogeneity in microglial density among different brain regions (Salter and Beggs 2014). The term 'microglia' was first introduced into the scientific literature almost a century ago (Pio del Rio-Hortega introduced the concept in 1932 for the publication Cytology and Cellular Pathology of the Nervous System, edited by Wilder Penfield (**Figure 1**)) but only in the last couple of decades their role has started to become clear (Kettenmann, Hanisch et al. 2011).

Figure 1.4.1.1: Photomicrographs from del Rio-Hortega (Kettenmann, Hanisch et al. 2011)

Microglia are first and foremost phagocytic cells. Their very active cytoskeleton gives them the ability to survey their surroundings, monitoring their microenvironment through pinocytosis (Nimmerjahn, Kirchhoff et al. 2005), affecting neuronal activity, synaptic connectivity and preserving homeostasis(Nayak, Roth et al. 2014). Microglial tissue surveillance in the healthy CNS is almost exclusively performed by their long, thin, and highly branched processes, which extend and retract at average velocities of around 2.5 μm/min (Lee, Liang et al. 2008, Madry and Attwell 2015). Many processes show highly motile protrusions forming bulbous endings that are very changeable in size and have even greater motilities of 4μm/min. In stark contrast to their fast moving processes, which span a territory of 60μm in cortex, their small somata (10 μm or less) are almost stationary. Their high process motility (as

well as their high cell density in the brain) allows microglia to scan the entire brain parenchyma once every few hours. Every microglial cell occupies a defined territorial domain, which does not overlap with neighboring microglia (Nimmerjahn, Kirchhoff et al. 2005).

Microglial cells have always been considered the macrophages of the CNS, capable of responding to any kid of pathological insult by assuming an "activated" phenotype while otherwise being "resting". In contrast with this view, these cells are now emerging as a unique population distinct from their peripheral cousins, underlying functions that go beyond ordinary immune functions.
With their ever moving ramifications, microglia transiently contact synapses as well as extrasynaptic terminals apposing both the dendritic spines and the astoglial perisynaptic clefts and thus microglia are able to influence and control brain homeostasis(Salter and Beggs 2014).

Initially thought to derive from circulating monocytes like meningeal, choroid plexus, and perivascular macrophages, fate mapping has actually revealed that microglia originate from precursors that leave the yolk sack on E8.5-E9.0, entering the neural tube through the bloodstream thus constituting a self standing lineage (ontogenesis of microglia is depicted in **Fig. 1.4.1.2**) (Kettenmann, Hanisch et al. 2011, Nayak, Roth et al. 2014, Salter and Beggs 2014).
Once they gain access to the brain, the yolk-sack-derived microglia remain throughout life since the population is maintained by self-renewal in the healthy CNS with little to no contribution from bone marrow macrophages (Ajami, Bennett et al. 2007). A wide array of microglia-specific mRNA and proteins have been identified characterizing gene networks peculiar to the cell type in the adult brain. Additionally, this characteristic transcriptional profile shows a stimulus dependent plasticity that is far more complex from the M1-M2 polarization observed in macrophages (Mills 2012) and rather than "activating", microglia are able to shift . Microglia do share some features with circulating monocytes and macrophages residing in other tissues such as the expression of surface markers, intracellular signalling pathways involved with innate immunity, and more importantly, the capacity to phagocytose cellular debris and release various types of pro-inflammatory mediators (Salter and Beggs 2014). Still, these two population remain distinct and distinguishable.

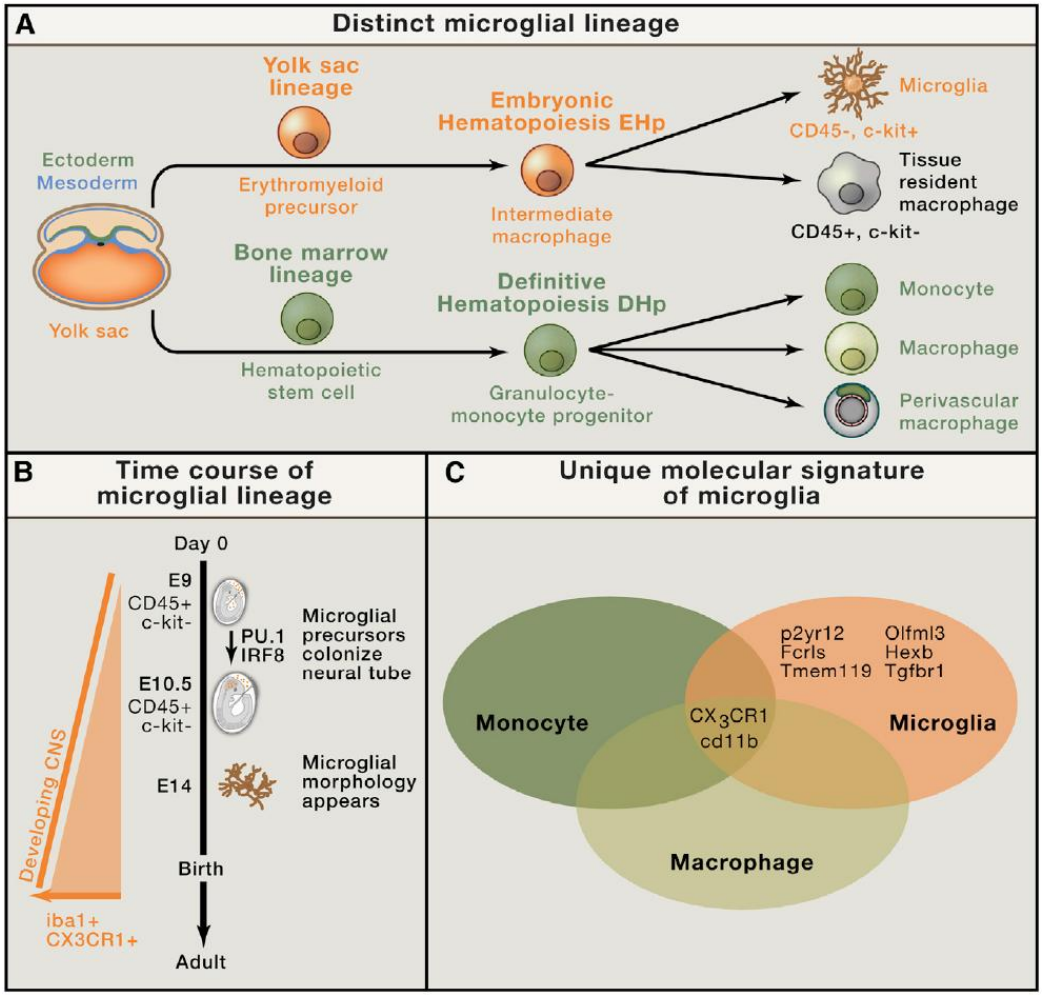

Figure 1.4.1.2: MICROGLIA HAVE A DISTINCT LINEAGE AND MOLECULAR SIGNATURE. (A) The genesis and progression of yolk-sac derived cell lineages are illustrated. Microglial are derived from primitive erythromyeloid progenitors in the yolk sac (embryonic hematopoiesis, indicated in yellow), distinct from the definitive hematopoiesis (shown in green) from which the majority of macrophages are derived. (B) An illustration of the time course of microglial derivation and embryonic colonization as revealed by fate-mapping experiments. Microglia originate from PU.1-dependent precursors in the yolk sac that proliferate and invade the neuroectoderm-derived developing CNS, as indicated by an increase in the markers CX3CR1 and iba1 (shown as orange ramp). (C) The diagram illustrates the overlap in gene expression between microglia, macrophages, and monocytes. Two of the most widely used ‘‘markers,’’ CX3CR1 and cd11b, are common to all three. Although there is considerable molecular similarity between tissue-resident macrophages and microglia, a subset of microglial-specific genes have been identified, and the six most prominent of these genes are shown (Salter and Beggs 2014).

In this picture, microglia stand out as far more than classical immune cells, and, as a matter of fact, increasing evidence has highlighted a variety of nonphlogistic functions of these cells in the normal healthy CNS.

1.4.2. THE ROLE OF THE MICROGLIA IN THE HEALTHY BRAIN

Microglia are indispensable for the normal brain development as they are involved in neuronal death and survival, synaptogenesis and the formation of the mature neuronal networks. For starters, microglia contribute to the developing brain by secreting trophic factors. As an example insulin-like growth factor-1 (IGF-1) released by surrounding microglia promotes survival of layer V cortical neurons during postnatal development (Ueno, Fujita et al. 2013). Microglia also secrete basic fibroblast growth factor, hepatocyte growth factor, epidermal growth factor, platelet-derived growth factor, nerve growth factor, and brain-derived neurotrophic factor, which play significant roles in neuronal development, maintenance, and function throughout life (Nayak, Roth et al. 2014).

The process of sculpting of the adult brain circuitry relies on programmed cell death of neurons and clearance of the resulting cellular debris. Microglia, as the main phagocyte of the brain, has of course a central role in this mechanism and it has been confirmed for many situations.

An example of this was observed in the retina of the developing chick eye, where release of nerve growth factor by microglia induced apoptosis of retinal nerve cells as part of the normal developmental program(Frade and Barde 1998). Likewise, in the developing murine hippocampus microglia induces neuronal programmed cell death(Wakselman, Bechade et al. 2008). Specifically, microglia induces cell death by releasing reactive oxygen species (ROS) in a CD11b-dependent and DNAX activation protein of 12 kDa (DAP12)-dependent manner.

As one could expect, microglia participate also in the cleanup through phagocytosis and, importantly, manage to do so without activating an inflammatory response(Takahashi, Rochford et al. 2005). Noninflammatory microglial phagocytosis of apoptotic neurons is mediated through signaling via triggering receptor expressed on myeloid cells-2 (TREM2). Dying neurons in turn release factors that recruit microglia to actively participate in phagocytosis. This phagocytic activity is important not only in the developing CNS, but also for the maintenance of healthy neural networks in the adult brain. For example, release of soluble fractalkine (CX3CL1) by damaged neurons can promote microglial phagocytosis through release of milk fat globule–epidermal growth factor 8 (MFG-E8) (Noda, Doi et al. 2011). Microglia are therefore important for removing cell bodies in the developing and adult CNS.

Furthermore, microglial phagocytic activity is involved in synaptic homeostasis. During postnatal development in mice, microglia are able to actively engulf synaptic material playing a major role in synaptic pruning. Microglia are therefore implicated in synaptic maturation and might contribute to synaptic abnormalities(Paolicelli, Bolasco et al. 2011).

Moreover, in organotypic hippocampal brain slice cultures microglia was able to modulate synaptic activity by regulating the density of synapses, spine numbers, surface expression of AMPA receptor (GluA1), and levels of synaptic adhesion molecules(Ji, Akgul et al. 2013).

In the visual cortex, light deprivation drives changes in microglial morphology, with microglial processes dysplaing phagocytic structures and apposing synaptic clefts more frequently. Light reexposure reversed these behaviors. These evidence shows that microglia can actively contribute to experience-dependent plasticity, modification and elimination of synapses in the healthy brain(Tremblay, Lowery et al. 2010).

Synaptic pruning seems to mediated in part by fractalkine signaling, as mice deficient in CX3CR1 had increased dendritic spine densities and less mature synapses a couple of weeks after birth(Paolicelli, Bolasco et al. 2011). The complement system also appears to be involved. During development, C1q localizes to synapses and can promote microglial phagocytosis through the complement receptor 3 (CR3) by activating the C3 cascade(Stevens, Allen et al. 2007).

This synaptic pruning is critical for establishing functional neuronal circuits and deficit in the process might be at the base of neurodevelopemental disorders such as autism(Nayak, Roth et al. 2014).

Moreover, because of their dynamic processes, microglia are in the right position to monitor neuronal firing activity and synaptic function, as they express receptors for numerous types of neurotransmitters and mediators (Kettenmann, Hanisch et al. 2011). In response to neuronal activity, microglia steer their processes toward active synapses, which facilitates contact with highly active neurons. Using live imaging in the mouse, microglia processes have been found to not only passively monitor neuronal activity, but also actively respond (Tremblay, Lowery et al. 2010, Wake, Moorhouse et al. 2013). Microglial processes in the optic tectum of zebrafish were seen to be attracted by neuronal activity and afterward this contact reduced both spontaneous and evoked activities of neurons(Li, Du et al. 2012).

Moreover, microglia also indirectly affect excitatory neurotransmission via an ATP-dependent mechanism that involves astrocytes(Pascual, Ben Achour et al. 2012)
Thus, microglia are not ‘‘resting’’ but are highly active in a ‘‘surveillance and rapid response’’ state in which they monitor and control the activity of neurons even in the healthy CNS.

1.4.3. MICROGLIA AND AD

The concept of a microglial involvement in AD arose primarily from the close spatial relation of these cells and Aβ plaques in AD brains (**fig.2**), an association so striking that initially even led to the speculation (now dismissed) that microglia might be the source of the deposited amyloid (Terry, Gonatas et al. 1964).

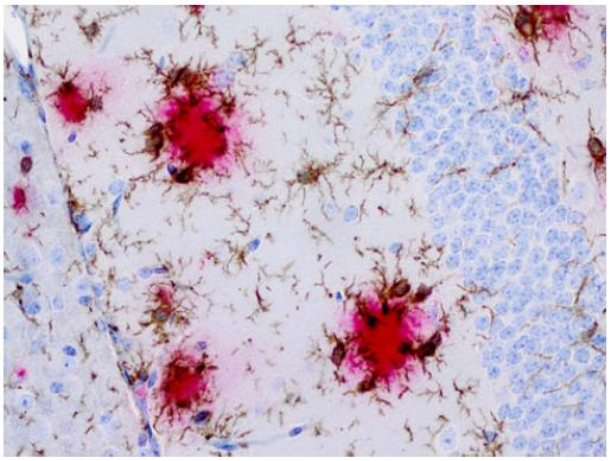

Figure 1.4.3.1: Microglia closely associated with b-Amyloid plaques. Activated microglia are clustering around a cerebral b-Amyloid plaque in a mouse model of Alzheimer’s disease (Iba1 immunohistochemistry, brown; 4G8 immunohisto-chemistry, red) (Prokop, Miller et al. 2013)

The interpretation of these cells in the context of AD has proven itself to be a difficult matter and the debate whether the microglial primary effect in neurodegeneration is detrimental or beneficial is still vivid and dynamic. Evidence indicates toward an “activated” phenotype of these cells through the detection of MHC-class II (HLA-DR) expression on microglia in the vicinity of Aβ plaques (Itagaki, McGeer et al. 1989). However, even though increased MHC-class II antigen expression has been correlated to activation per se (Akiyama, Itagaki et al. 1988, Smetanka, Yee et al. 1990), the expression of this marker is far from being limited to activated microglial cells (Hayes, Woodroofe et al. 1987). Moreover while morphological changes have been and are still repeatedly used to characterize activation (Streit, Walter et al. 1999), this approach has its limits, starting from being able to detect only deep functional changes.

It is this actual absence of markers, of precise and defined phenotypes of microglia(that is mostly due to gaps in the knowledge of their physiology), that hinder the

identification of the functional activity states of these cells and make it difficult to pinpoint their role in the pathogenesis of AD.

"Activated microglia" is a broad term that includes each and every one of the responses to chemical or cell based cues that microglia adopts and that cannot be expressed by a limited number of cell-surface antigens or morphological appearance. Microglial physiology is way past the M1-M2 polarization hypothesized first for macrophages where there is a dichotomic distinction between classically activated via toll-like receptors or interferon γ (M1) versus alternatively activated by interleukin 4 or interleukin 13 (M2) (Martinez, Sica et al. 2008). Activation has always been considered a one way street where the microglial cell from resting and extensively ramified retracts all of their processes and acquire an "ameboid" activated form, the only form deemed capable of reacting(Dheen, Kaur et al. 2007).

In reality microglial responses exist as a spectrum, a continuum of functional states able to show plasticity to both signals and context (Perry, Nicoll et al. 2010). Phenotype is ultimately flexible and to more appropriately evaluate microglial roles in health and disease it is thus required a more nuanced and sophisticated understanding of the complexity of such phenotypes.

1.4.4. THE GOOD, THE BAD AND THE MANY FACED MICROGLIA

Given these premises and observations, the unresolved question of whether microglial responses are detrimental or beneficial remains.

Studies in AD patients samples have shown increased microglial release of immune molecules such as interleukin (IL)-1 (Griffin, Stanley et al. 1989), IL-12 and IL-23 (Vom Berg, Prokop et al. 2012) or complement proteins (McGeer, Akiyama et al. 1989), while numerous more proinflammatory, toxic molecules have been demonstrated to be increased either in expression or secretion in vitro upon stimulation with Aβ, including many cytokines, chemokines and growth factors like tumor necrosis factor (TNF)a, IL-1b, IL-6, macrophage inflammatory protein (MIP)1a, macrophage chemotactic protein (MCP)-1, IL-8, macrophage-colony stimulating factor (M-CSF) and IL-12 (Prokop, Miller et al. 2013). This evidence led to the view of microglia as neurotoxic cells aiding the neurodegeneration(Madry and Attwell 2015).

Nevertheless, there are numerous studies advancing a beneficial role for the microglia, studies that demonstrate the secretion of neuroprotective cytokines like transforming growth factor β1(TGFβ1) as well as reports of worsened pathology when microglia are inhibited (Prokop, Miller et al. 2013). An interesting study performed in vivo showed that microglia is actually able to constitute a barrier around plaques with profound impact on plaque composition and toxicity (Condello, Yuan et al. 2015). For starters, it appears that areas uncovered by microglia are less compact but have high Aβ42 affinity, leading to the formation of protofibrillar Aβ42 hotspots, regions that are actually associated with more severe axonal dystrophy. Interestingly during ageing, this microglia coverage is indeed reduced leading to an enlargement of these hotspots.

This amyloid-driven activation that characterizes microglia has led to the use of the term "neuroinflammation", one usually associated with encephalitis, cerebral vasculitis or multiple sclerosis and that entails acute brain inflammation. The main difference between AD and these primarily neuroimmune disorders is that in the latter peripheral immune cells, specifically T and B cells, act as key players of the immune reaction in the brain. Conversely, such infiltrates are virtually absent in human AD (Streit 2004).

The picture depicted from the in vitro studies led to a modification of the amyloid hypothesis that included a positive feedback loop where activated microglia would secreare proteases cytokines and ROS inducing more Aβ synthesis and accumulation that in turn activated further the microglia(Cherry, Olschowka et al. 2014).

The high levels of inflammatory cytokines measured in the cerebrospinal fluid and in post mortem analysis of the cortex of AD patients plus the in vitro studies demonstrating pro-inflammatory responses to Aβ have thus skewed the field toward limiting neuroinflammation instead of harnessing its power. However, this idea was proven wrong with the crushing failure of non-steroidogenic anti-inflammatory drugs in clinical trials showing that blocking all forms of inflammation is highly detrimental to patients, even accelerating disease progression in some cases. In mouse models of AD, systematic gene deletion of key signaling molecules of the innate immune system has almost always resulted in worsening of symptoms, augmenting amyloid deposition and cognitive decline (Lampron, Elali et al. 2013) plus anti-inflammatory cytokines have been linked to a worsening of the AD phenotype (Chakrabarty, Li et al. 2015).

For what concerns, the major discrepancies between the microglial activation reported in vitro and the physiological situation is that in vitro experiments tend to use the "sledgehammer method" to achieve maximal stimulation, meaning high doses of LPS, Aβ, etc.. but at the same time it is documented that the strength of microglial activation in the normally aged and AD brain is consistent with only minimum neuroinflammation (Streit, Xue et al. 2014). It is thus important as ever to work in vivo while modulating microglia.

Neuroinflammation has been exclusively linked to Aβ, representing a mere epiphenomenon, the brain's way to respond to Aβ accumulation. In fact according to the amyloid hypothesis, immune-system activation follows Aβ deposition. Recently though, preclinical, genetic and bioinformatic data have shown that immune involvement actually accompanies the pathogenesis of this disease.

For starters the presence of inflammatory changes seem to precede clinical symptoms such as mild cognitive impairment (MCI) (Tarkowski, Andreasen et al. 2003, Brosseron, Krauthausen et al. 2014). In one study (Krstic, Madhusudan et al. 2012) it was demonstrated that systemic immune challenge was able to drive an AD-like pathology in a WT mouse (Cunningham, Campion et al. 2009). These observations suggest that microglia, as the primary immune mediator in the CNS, might in some way be capable of driving AD pathology independently of Aβ deposition.

The complicated picture of microglia in AD has been cleared with the following interpretation, though not accepted entirely: microglia reactivity in early stages of amyloid deposition emerges as beneficial to prevent plaque formation and to promote removal of existing Aβ deposits, while microglia-mediated inflammation in later stages of AD appears to be rather detrimental and may even serve to promote tau pathology(Prokop, Miller et al. 2013).

1.4.5. MICROGLIA AND Aβ

As garbage men of the brain, microglia have been shown to be able of phagocyting Aβ thus representing a key mechanism against Aβ accumulation in the brain(Frackowiak, Wisniewski et al. 1992, Frautschy, Cole et al. 1992, Shigematsu, McGeer et al. 1992, Weldon, Rogers et al. 1998).

Phagocytosis of *fibrillar Aβ* can occur through distinct pathways, primarily direct receptor binding, opsonizations by complement or immunoglobulin and interaction with chaperone molecules such as apolipoproteins. These molecules that have the ability to mediate the uptake of Aβ, have also been associated with AD through GWAS including complement receptor 1, apolipoprotein E and J, and phosphatidylinositol-binding clathrin assembly lymphoid myeloid leukemia protein (PICALM) (Harold, Abraham et al. 2009).

There are several receptors that can bind Aβ, allowing its recognition and uptake, as well as relay signals to modulate the inflammatory response (Sokolowski and Mandell 2011).
Notably, CD36 (Bamberger, Harris et al. 2003), class A scavenger receptor (El Khoury, Hickman et al. 1996) and receptor for advanced glycosylation end products(RAGE) (Du Yan, Zhu et al. 1997), all demonstrated the ability to activate microglia through interaction with Aβ.

The complement pathways appears to also be involved in the Aβ clearance process. In vitro experiments have suggested that complement-dependent phagocytosis is inhibited by proinflammatory cytokines and this might explain with in inflamed AD brains, clearance is insufficient (Koenigsknecht-Talboo and Landreth 2005). In hAPP transgenic mice, complement C3 deficiency caused by overexpression of a complement inhibitor, soluble complement receptor-related protein y (Scrry), or by knockout, results in increased Aβ plaque deposition (Wyss-Coray, Yan et al. 2002, Maier, Peng et al. 2008).

Moreover, natural antibodies to Aβ exist in the bloodstream and either passive or active immunization against Aβ appears to promote clearance (Schenk, Barbour et al. 1999). Antibodies seem to localize with plaques in vivo and to opsonize Aβ in vitro (Wilcock, Rojiani et al. 2004), though the reason why natural antibodies fail to clear plaques is still not clear. Different antibody avidity and epitope specificity, though, might underlie individual susceptibility to disease.

In addition, chaperone molecules such as apoliproteins have a significant role in Aβ clearance. For example, known primarily for its role in cholesterol homeostasis, ApoE appears to modulate Aβ uptake by forming Aβ-APoE complexes that are then

recognized by receptors on the cell surface such as LRP (low density lipoprotein receptor related protein) and LDLR (low density lipoprotein receptor). Binding subsequently stimulates clathrin mediated endocytosis. Evidence supporting a disruption of these mechanism comes from the genetics since ApoE was identified through GWAS as a major risk factor for LOAD.

These molecules show a higher affinity for the fibrillar form of Aβ, while the *soluble* form(the most toxic one) was reported to be cleared by microglia through fluid phase macropinocytosis (Mandrekar, Jiang et al. 2009), a process that needs no receptors and constitutes an aftermath of the microglial ever constant sampling of the extracellular environment and it is mostly dependent on the highly dynamic actin cytoskeleton these cells have.

If we consider that Aβ accumulation is clearly caused by an imbalance between Aβ production and clearance, and that clearance rates for both Aβ42 and Aβ40 are impaired in AD compared with controls while no differences can be demonstrated in Aβ40 or Aβ42 production rates(Mawuenyega, Sigurdson et al. 2010), then microglial impairment in Aβ clearance is one of the significant drivers of pathophysiology in sporadic AD.

Interestingly, senescent changes in microglia have been proposed to develop with age, including a loss of dynamicity and plasticity of the microglial actin cytoskeleton (Flanary, Sammons et al. 2007, Njie, Boelen et al. 2012, Uhlemann, Gertz et al. 2015). With these considerations in mind, we can imagine a gradual loss of the Aβ clearance power of microglia as a natural consequence of age, a tendency whose steepness is influenced by genetic and environmental factors and that can result in pathology once Aβ toxicity kicks in.

1.5. MICROGLIA AND NGF

Not much literature exist on the role of NGF in microglial physiology. Microglia have been demonstrated to be capable of secreting NGF and also responding to it by means of both TrkA and p75NTR(Nakajima, Kikuchi et al. 1998, Kettenmann, Hanisch et al. 2011).

Interestingly, NGF was shown to influence microglial motility by exerting chemotactic activity, a response that is TrkA dependent sicce it was prevented by specific inhibitors

of TrkA kinase activity or MAPK/ERK cascade (De Simone, Ambrosini et al. 2007). LPS-induced activation of microglia can trigger the expression of NGF in cultured microglial cells, which can also be induced by stimulation of A2 adenosine receptors (Heese, Fiebich et al. 1997). Concerning activation, NGF could also decrease MHC class II inducibility in microglia underlying some sort of anti-inflammatory effect of the neurotrophin (Neumann, Misgeld et al. 1998).

NGF and microglia are also partners in the sculpting of the developing retina. Indeed, microglia cells were shown to be the source of NGF that induces neuronal death through p75 signaling pathways in the developing eye (Frade and Barde 1998, Srinivasan, Roque et al. 2004).

Moreover, NGF treatment of macrophages and microglia significantly increased the levels of cathepsin S, B, and L mRNAs (2- to 5-fold). Cathepsins are members of the family of cysteine lysosomal proteases and some of them have been demonstrated capable of digesting Aβ (Liuzzo, Petanceska et al. 1999).

In the world of peripheral phagocytes, the link NGF-phagocytosis has been far more investigated so information about the supposed though not confirmed role of this neurotrophin on microglia can be drawn from these cells.

In macrophages, NGF promotes membrane ruffling, calcium spiking and growth factor secretion(Williams, Killebrew et al. 2015) and also phagocytosis (Susaki, Shimizu et al. 1996). Moreover, NGF has been linked to survival, phagocytosis, and superoxide production in murine neutrophils (Kannan, Ushio et al. 1991). Because of this evidence, it is very likely that NGF exert the same action on phagocytosis in microglia as the one observed in their peripheral cousins.

1.6. TREM2: A NEW PROTEIN OF INTEREST FOR AD AND MICROGLIA

One of the biggest revelation when it comes to the role of microglia in AD was the identification of the association between Alzheimer and the mutations in the gene encoding the triggering receptor expressed on myeloid cells 2(TREM2)(Jonsson, Stefansson et al. 2013) and other microglia-related genes. This conceptual transformation in the field was due to the fact it was the first and only evidence of a role of the immune system in AD pathogenesis beyond the purely descriptive level. This link was later reinforced when TYROBP/DAP12, the gene encoding the TREM2 adaptor

protein, was found to be strongly associated with the pathophysiology of late onset AD (LOAD) as a key network regulator (Zhang, Gaiteri et al. 2013).

TREM2 (Bouchon, Dietrich et al. 2000) is a 40-kDa type I membrane glycoprotein with a single extracellular immunoglobulin-like domain, one trans-membrane domain, and a short cytoplasmatic tail. Although the cytoplasmic domain of TREM2 does not contain an overt amino acid-based signaling motif, its transmembrane domain interacts with the adaptor protein TYROBP/DNAX-activating protein of 12 kDa (DAP12) via electrostatic interaction(Colonna 2003). TREM2 was originally identified on monocyte-derived dendritic cells, but was later also detected on several myeloid cell types including osteoclasts, tissue macrophages, and microglia. TREM2 is localized predominantly in the Golgi complex, but also shuttles to and from the cell surface in endocytic and exocytic vesicles (Sessa, Podini et al. 2004, Prada, Ongania et al. 2006). DAP12 is a type I transmembrane protein, which acts as a signaling adaptor protein for TREM2 and a number of other cell surface receptors. The cytoplasmic domain of DAP12 contains an immunoreceptor tyrosine activation motif (ITAM)(Colonna 2003). After activation of the interacting receptor, DAP12 undergoes phosphorylation at the two conserved ITAM tyrosine residues by Src kinases. Subsequent recruitment and activation of the Syk protein kinase trigger downstream signaling pathways, including the activation of mitogen-activated protein kinase (MAPK) and phospholipase Cγ (PLCγ) (Lowell 2011). At present, heat shock protein 60 (HSP60) is the only ligand identified for activating TREM2-mediated phagocytosis of apoptotic cells (Stefano, Racchetti et al. 2009).

Interestingly, TREM2 undergoes sequential proteolytic processing by ectodomain shedding and intramembrane proteolysis and the C-terminal fragment (CTF) of TREM2 generated by ectodomain shedding appears to be cleaved by the γ-secretase (Wunderlich, Glebov et al. 2013), supplying yet another link with AD.

The physiological relevance of TREM2 and DAP12 is highlighted also by loss-of-function mutations of either gene that cause polycystic lipomembranous osteodysplasia with sclerosing leukoencephalopathy (PLOSL or Nasu-Hakola disease) associated with presenile dementia in the fourth decade of life in homozygous carriers (Paloneva, Kestila et al. 2000, Paloneva, Manninen et al. 2002).

TREM2 seems to be involved in two signaling pathways in microglia: phagocytosis and proinflammatory cytokines secretion.
Indeed, increased expression of TREM2 on microglia has been associated with enhanced phagocytosis and increased "alternatively activated M2" protective microglia, while absence of TREM2 expression on microglia does exactly the opposite, impairing phagocytosis and increasing the proinflammatory phenotype (Neumann and Takahashi 2007, Takahashi, Prinz et al. 2007). TREM2 phagocytic function is not associated with inflammation. Instead, it suppresses inflammation and promotes tissue repair. This has been supported by the evidence from animal models of multiple sclerosis (Piccio, Buonsanti et al. 2007, Takahashi, Prinz et al. 2007). In these studies, blockade of TREM2 binding with ligand by a monoclonal antibody worsened disease, whereas overexpressing TREM2 in microglia improved it. It has also been shown that transduced TREM2 expression in microglia prevented induction of the pro-inflammatory cytokine tumor necrosis factor-α and nitric oxide synthase 2 transcripts (Takahashi, Rochford et al. 2005).

Several rare variants of TREM2 in the heterozygous state have recently been identified by exome sequencing to increase the risk of late onset Alzheimer disease (LOAD) (Benitez, Cooper et al. 2013, Guerreiro, Wojtas et al. 2013).

This risk factor is nothing but negligible. In fact, one particular TREM2 variant, R47H, though not so frequent in the population, has an effect on individual risk of developing AD comparable with the apolipoprotein ε4 allele (Guerreiro, Wojtas et al. 2013, Jonsson, Stefansson et al. 2013)**.**

TREM2 was also found to be up-regulated in microglia of amyloid precursor protein transgenic mouse models (Frank, Burbach et al. 2008, Melchior, Garcia et al. 2010). Moreover, in post-mortem temporal cortices of AD patients, intense TREM2 immunoreactivity was observed in the microglia associated with amyloid plaques and in neuritic pathology-enriched areas (Lue, Schmitz et al. 2015). These data can be interpreted as a way for the brain to try and eliminate plaques.

Given its positive correlation with amyloid phagocytosis and overall non-inflammatory nature, impaired microglia TREM2 signaling is likely to result in decreased Aβ clearance and enhanced inflammation in AD patients harboring mutations in this gene.

AIMS OF THE RESEARCH THESIS

Several open questions in AD11 biology remain. One of them is what cell type mediates the phenotype (or even IF that could be narrowed down to just one specific population, since such a phenotype is bound to be cell autonomous up to certain length). I addressed the question of microglial involvement in the AD-like phenotype of this peculiar mouse model, focusing in the presympthomatic and early sympthomatic phases (1 and 6 months) proposing a microglial impairment due to NGF deprivation. Since already at one month changes in the AD11 transcriptome are already detected (changes related to immunity in particular- see chapter 1.3.1 THE AD11 MOUSE MODEL), I analyzed microglia starting from that time point. The 6 month time point represents the beginning of the Aβ accumulation and behavioral impairment though without the neuronal cell loss associated in AD with the proinflammatory-activated (potentially neurotoxic) microglial phenotype. So it is in this time frame that changes of the homeostasis of microglia might be able to induce the pathological framework of AD, should be detected.

The main points of my master thesis are:

- Characterization of microglial phenotype in AD11 through a classical morphological approach but with specific parameters directed towards a more subtle evaluation of phenotypic changes in microglia.
- Assessment of TREM2 levels in AD11 to assess microglial phagocytic impairment in the mouse model of AD.
- Mimic NGF deprivation in vitro in cell cultures treated with αD11 antibody and assessing TREM2 transcriptional and protein levels to confirm a possible link between the decrease in microglial phagocytic ability, NGF deprivation and AD-like phenotype.

MATERIAL AND METHODS

1. ANIMALS

For IHC and WB analysis, AD11 mice (Ruberti, Capsoni et al. 2000), 1 and 6 months old, were used. Littermates were used as controls for the IHC, since AD11 has a C57BL/6 x SJL background. C57BL/6 mice were used to obtain primary microglial cultures. AD11 x CX3CR1-GFP $^{+}$ mice were used for the isolation of microglia from adult brain (ongoing project - FUTURE AND PERSPECTIVES) and FACS analysis. Genotyping was performed by PCR analysis of tail DNA. All experiments with mice were performed according to the national and international laws for laboratory animal welfare and experimentation (EU directive n. 2010/63/EU and Italian DL n. 26 04/03/2014). Mice were kept under a 12-h dark to light cycle, with food and water *ad libitum.*

2. TISSUE PROCESSING FOR HISTOLOGICAL AND BIOCHEMICAL EXPERIMENTS

For IHC analysis, mice were anaesthetized with 2,2,2-tribromethanol (400 mg/kg) and intracardially perfused first with PBS (Phosphate Buffered Saline; NaCl 137 mM, KCl 207 mM, Na_2HPO_4 10mM, KH_2PO4 1.8 mM) and then with a 4% solution of paraformaldehyde (always in PBS). Brains were collected and post-fixed in the same solution for at least 4 hours. Brains were transferred in 30% sucrose/PBS solution and then sectioned at a sliding freezing microtome (Leica, Wetzlar, Germany). Forty micrometers sections were collected in 0.05% sodium azide/PBS in 1.5 ml tubes and stored at 4°C until usage.

BRAIN SAMPLE PREPARATION FOR WESTERN BLOT

Lysates for WB analysis were prepared as follows:

- Cortex was dissected with surgical instruments and placed on ice to slow down degradation due to protease activity
- Tissue was kept at -80°C until usage
- Tissue was homogenized through sonication keeping it on ice and centrifuged 30min, 13.000 RPM at 4°C
- The supernatant was aliquoted and kept at -80°C

- A Bradford assay was performed to determine protein concentration using Bovine Serum Albumin (BSA) for the standard curve.

3. MICROGLIAL CULTURES

The immortalized BV-2 murine microglial cell line (Henn, Lund et al. 2009) will be grown and maintained in DMEM containing penicillin/streptomycin 1% and 10 % Fetal Bovine Serum (FBS) at 37 °C in 5% CO2. Mixed cultures of microglia and astrocites were prepared from brains of P1-P4 mice C57BL/6J mice (Bronstein, Torres et al. 2013) Briefly, animals were sacrificed, the brains were removed in ice-cold PBS buffer containing 100 units penicillin and 0.1 mg/ml streptomycin, the cortex was dissected and collected in the same buffer. Cortices were quickly minced with tweezers and cellularized in 0.25% trypsin solution in the same buffer. After 10-15 minutes in the incubator at 37°C, trypsin activity was stopped by adding an equal volume of DMEM/F-12(Dulbecco Modified Eagle Medium, Nutrient Mixture F12; Life Technologies) + 10% FBS and 5 mg/ml DNAse (Life Technologies). After a 1200 RPM centrifugation for 5 minutes, cells were collected and resuspended in culture medium (DMEM/F-12, 100 units penicillin, 0.1 mg/ml streptomycin, Glutamax 1X + 10% fetal bovine serum). After 14 day of culture, microglia was mechanically dissociated to be plated for experiments.

4. MICROGLIA ISOLATION FROM ADULT MOUSE BRAIN

Microglial isolation from 1month animals was performed as shown by Lee and Tansey (Lee and Tansey 2013) with a protocol that involves enzymatic dissociation in medium supplemented with dispase II, papain, and DNase I followed by mechanical dissociation. Cell separation was achieved via Percoll gradients of various densities. Five animals from the genotype AD11 x CX3CR1-GFP $^{+}$ 1 were used to obtain fluorescent microglia.

MATERIALS

- PBS-perfused brains from adult mice.
- Hank’s balanced salt solution (HBSS) without calcium and
- magnesium.

- 10× HBSS.
- DNase I (final concentration; 20 U/mL, Invitrogen).
- Dispase II (final concentration; 1.2 U/mL, Roche).
- Papain (1 mg/mL, Sigma-Aldrich).
- DMEM/F12 medium.
- 100× penicillin/streptomycin.
- Percoll.

MEDIA AND SOLUTIONS

- Dissection medium (50 mL): 49.5 mL of Hank's Balanced Salt Solution (HBSS) plus 0.5 mL of penicillin/ streptomycin(100×). All solutions were sterile filtered after mixing/before use.
- Glucose: Prepare 0.45 g/mL in PBS (100×). Add 500 μL in 50 mL reagents to make final concentration of 4,500 mg/L.
- Serum-free medium (50 mL): 49 mL of DMEM/F12 plus 0.5 mL of penicillin/ streptomycin (100×) and 0.5 mL of glucose (100×). All solutions were sterile filtered after mixing/before use.
- Neutralization medium (50 mL): 44 mL of DMEM/F12 plus 0.5 mL of penicillin/streptomycin (100×), 0.5 mL of glucose (100×), and 5 mL of heat inactivated FBS (or FCS). All solutions were sterile filtered after mixing/before use.
- Culture medium (50 mL): 44.5 mL of DMEM/F12 plus 0.5 mL of penicillin/streptomycin (100×) and 5 mL of heat-inactivated FBS (or FCS). All solutions were sterile filtered after mixing/before use.
- Dispase II: Dissolved the non-sterile lyophilized enzyme in HEPES buffered saline (50 mM HEPES/KOH pH 7.4, 150 mM NaCl) (10 mg/mL). The solution was diluted further with the culture medium to be used for the isolated cells at the final concentration of 2.4 U/mL. Sterilized through a 0.22 μm filter membrane.
- Dissociation medium: dispase, DNase, and papain (DDP) solution was prepared.
    - ✓ 0.028 g papain (fi nal concentration 1 mg/mL).
    - ✓ 14 mL of DMEM/F12.

- ✓ 14 mL premade dispase II (fi nal concentration 1.2 U/mL).

Aliquoted and stored at −20 °C.

Add DNase I (fi nal concentration 20 U/mL) right before use.

- Percoll preparation: stock isotonic percoll (SIP) was prepared; nine parts of percoll with one part 10× HBSS were mixed.
  - ✓ 70 % percoll: 7.0 mL of SIP plus 2.0 mL of 1× HBSS.
  - ✓ 37 % percoll: 3.7 mL of SIP plus 5.3 mL of 1× HBSS.
  - ✓ 30 % percoll: 3.0 mL of SIP plus 6.0 mL of 1× HBSS.

METHODS

1. The freshly perfused adult brain were placed in 2 mL serum-free media into the 35 mm dish and minced the freshly perfused adult brain as finely as possible using the 15 T scalpel blade. The amount of serum-free medium was made sure to be equivalent to the amount of dissociation medium.
2. Minced brain were transferred to 15 mL tube containing 3 mL of dissociation medium.
3. Cell suspension was gently rocked (or inverted the tube every 5 min) in tissue culture incubator for 20 min.
4. The enzymes was neutralized in dissociation media by adding 5 mL of neutralization medium.
5. Cell suspension was centrifuged 5 min at 250 × *g* in room temperature.
6. The medium was slowly aspirated slowly (being extremely cautious not to disturb the pellet as it can easily be aspirated by the aspirating pipette).
7. The pellet was resuspended in 5 mL of serum-free medium. **Steps 5** and **6** were repeated.
8. 3 mL DMEM/F12 were added and pipetted up and down with polished *large size-hole Pasteur* pipette against bottom of tube until large clumps of tissue were broken up. We waited for 1 min until large clumps settled. The upper part (containing dissociated cells) was transferred into new 15 mL conical tube and kept it on ice.
9. 3 mL of DMEM/F12 were added and pipetted up and down with polished Pasteur pipette (*medium-size hole*) against bottom of tube until large clumps of tissue are broken up. We waited for 1–2 min until large clumps settle. The upper

part (containing dissociated cells) was transferred into new 15 mL conical tube and kept it on ice.

10. 2 mL DMEM/F12 were added and pipetted up and down with polished Pasteur pipette (*small-size hole*) to break clumps. We waited for 1 min until large clumps settle.
11. The upper part (containing dissociated cells) was combined into previous cell suspension.
12. A 40 μm cell strainer was wetted with 2 mL of DMEM/F12 and then used to filter the cell suspension.
13. Suspension was spinned at 250 × *g* for 4 min.
14. Cells were resuspended in 5 mL DMEM/F12, spin 250 × *g* for 4 min, and supernatant was removed.
15. Cell pellet was resuspended in 4 mL per brain of 37 % SIP.
16. 4 mL of the 37 % SIP (from **step 15**) was transferred to 15 mL conical tubes, slowly underlying 4 mL of 70 % percoll (*see* **Note 1**). Then on top of the 37 % layer, 4 mL of 30 % percoll was slowly pipetted, followed by 2 mL of HBSS (**Fig. 4.1**).

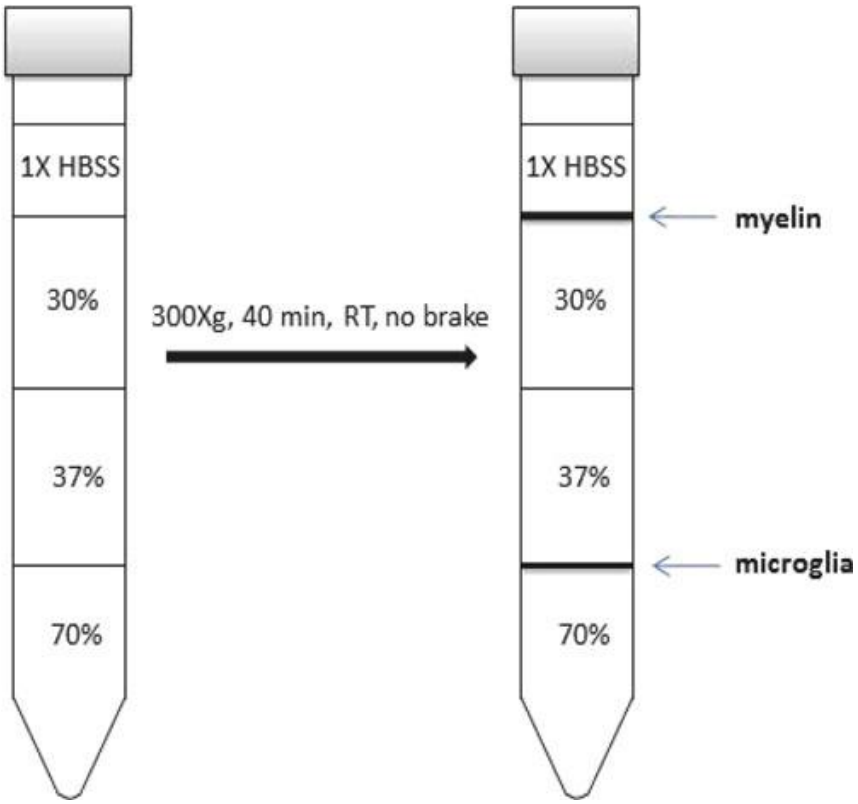

Figure 4.1: Schematic of the percoll gradient setup for isolation of adult mouse microglia (Lee and Tansey 2013).

17. Gradient was centrifuged for 40 min at 300 × *g* (18 °C) with no brake so that the interphase would not be disturbed.
18. Using a transfer pipette, the layer of debris was removed and 2.0–2.5 mL of the 70–37 % interphase was collected into a clean 15 mL conical tube.
19. 6 mL of HBSS for each 2 mL of interphase volume collected was added to ensure the percoll containing the interphase would be diluted about three times.
20. We centrifuge for 7 min at 500 × *g* at 4 °C.
21. Pellet was resuspended in 500 μL of HBSS and transfered to small 0.6 mL or 1.5 mL tubes and washed three times in a volume of 500 μL, using a micro-centrifuge at 800 × *g* at 4 °C.
22. Cells were counted and processed them for western blotting by digesting them with RIPA buffer, brief sonication, centrifugation 15min 4°C and storing them at -80°C or resuspended in PBS for FACS analysis.

5. TREATMENTS WITH αD11 FOR RT-PCR AND WB

Microglial cells, detached mechanically from the mixed culture, or BV-2 microglial cells were plated at a density of $5x10^5$ cells/well in a six-well plate overnight in DMEM containing 10% FBS. The day after medium was replaced with: DMEM-F12-10% FBS+800ng/ml mAb αD11 or DMEM-F12-10% FBS+800ng/ml anti v5 (as a control) or DMEM-F12-10% FBS as a blank. The Anti v5 (Sigma-Aldrich) is an antibody produced in rabbit that recognizes a tag and because of its specificity for a synthetic peptide (which will not be present in mouse) was chosen as a control antibody. Treatment with these antibodies lasted 48h. At the end of the 48h the medium was removed, cells were washed with cold PBS and were either lysed using RIPA buffer (10mM Tris-Cl(pH 8.0), 1mM EDTA, 0.5 mM EGTA, 1% triton X-100, 0.1% sodium deoxycholate, 0.1% SDS, 140 nM NaCl) containing protease inhibitor cocktail (Sigma-Aldrich) for WB analysis or collected and stored at -80°C for RT-PCR. For WB, after using the RIPA buffer cells were briefly sonicated and centrifuged 15min at 4°C to remove the unsoluble fraction. Protein content concentration was measured through a Bradford assay.

6. IMMUNOHISTOCHEMISTRY

Morphologic assessment of microglial condition was performed through Iba1 immunostaining as described previously (Kettenmann, Hanisch et al. 2011, Torres-Platas, Comeau et al. 2014) in 1 and 6 month-old mice AD11, VH, WT (N= 3 to 6 animal per group). The VH phenotype was used as an internal control.

PROTOCOL

The cortical sections were treated as follows:

- ✓ Washed 3 times for 10 min in Tris-Buffered Saline+TritonX 0.3% (TBST) (Tris-Buffered Saline: 50mM Tris-Cl, ph 7.5; 150mM NaCl)
- ✓ 30 min. in TBS+H_2O_2 3.5%
- ✓ Washed again 2 times for 10 min in TBST
- ✓ 30 min. pre-incubation in FBS (TBST+10% FBS), RT.
- ✓ Incubation overnight with anti-Iba1 (1:100; rabbit; Wako) in 10% FBS, at 4 C°
- ✓ Washed 3 times for 10 min in TBS
- ✓ Incubated 3h with secondary AlexaFluor488 anti rabbit (1:100, Invitrogen) in 10% FBS in TBS, RT.
- ✓ Washed 2 times for 10 min in TBS
- ✓ Mounted on gelatin-coated slides with fluoromount adhesive (Sigma-Aldrich, St. Louis,Mo).

CONFOCAL MICROSCOPY AND IMAGE ANALYSIS

Images from AD11, VH and WT mice were acquired with a confocal laser scanning microscope (TCS SP2; Leica Microsystem, Wetzlar, Germany). ~10 stacks per region per mice were obtained. On the Z axis, images were taken every 0.4 µm(axial resolution was determined with the formula $r_{axial} = 1.4\lambda \bullet \eta / NA^2$ where λ is the emitted light wavelength, η is the index of refraction and NA is the numerical aperture of the objective- in our case it was a 64x oil). Regions of interest for morphological changes in the microglia were the enthorinal cortex and the hippocampus(specifically the molecular layer)(**Fig.1**). These regions were chosen because of their association with AD in general and with AD11specifically . Image elaboration and analysis was conducted using Bitplane's software Imaris (**Fig.2)** (Zurich, Switzerland).

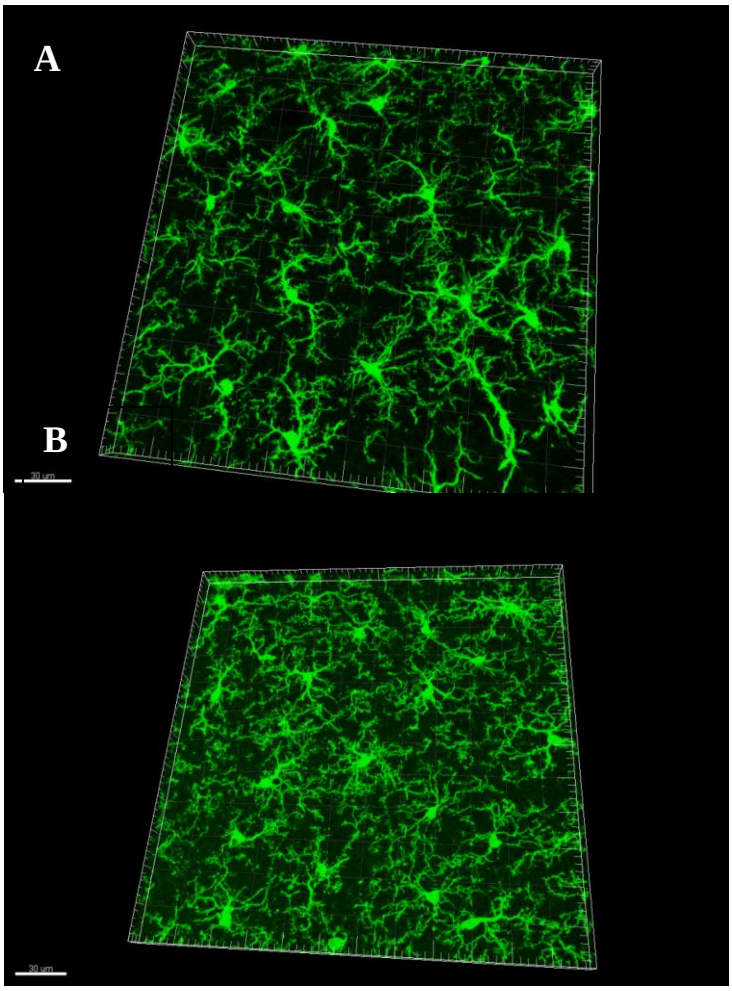

Figure 6.1: stacks obtained from a WT mice, showing extensive Iba1 positive ramifications all over the hippocampus(**A**) and the cortex(**B**).

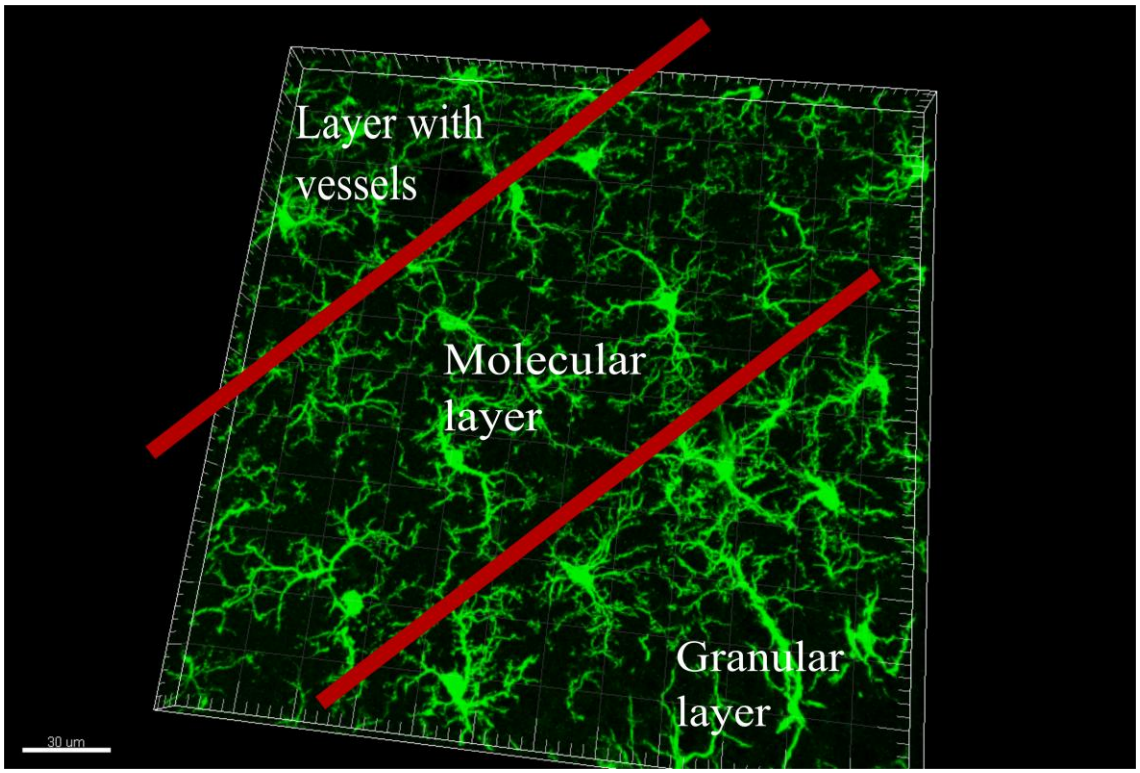

Figure 6.2: selection of the ROI in the hippocampus.

The “filament” function, was used to reconstruct the exact shape and length of the cell processes, while the “volume” function was used to determine the volume of the slice. Parameters extrapolated from the software were filament length per cell, number of branching points per cell, volume of the entire slice, number of cells per slice.

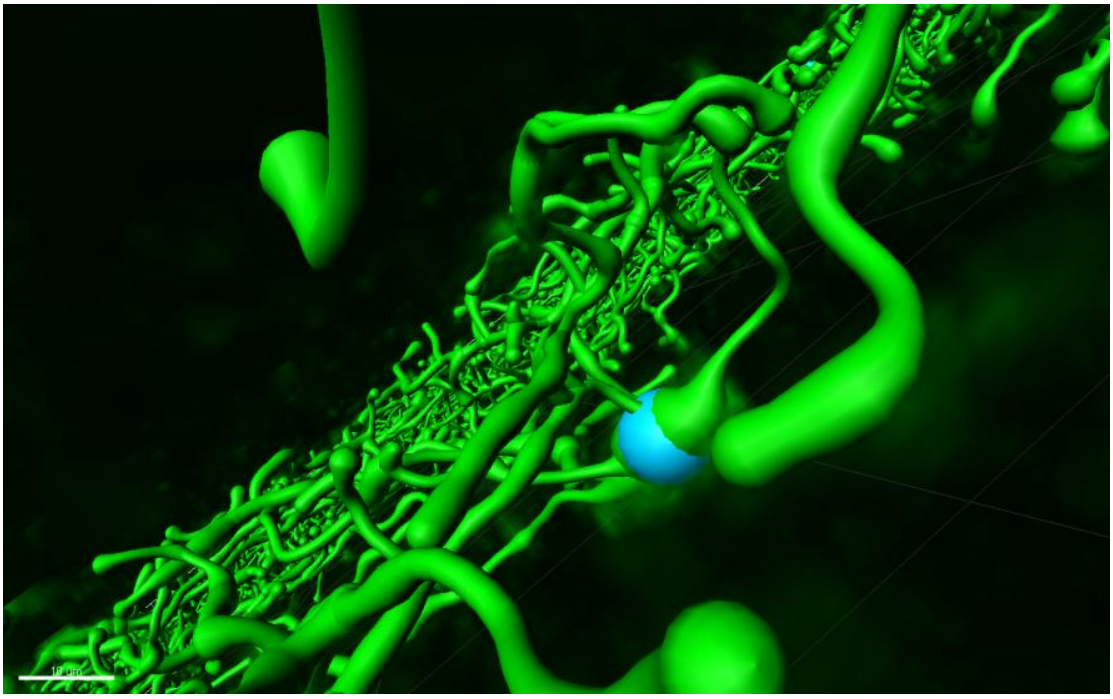

Figure 6.22: Inside the IHC: Image taken with Imaris (Zurich, Switzerland). Blue spheres are microglial nuclei. Green filaments are the microlial processes.

7. IMMUNOCYTOCHEMISTRY(ICC) TO DETECT IBA1 AND TREM2

Immunocytochemistry was performed to get a visual representation of TREM2 expression on primary cultures.

Primary murine microglia were plated on coverslips in 24-well plates at a density of $1x10^5$ cells/well for 18 h. The media was removed and replaced with DMEM/F12 either plain or in conjunction with mAb αD11 or αv5(800 ng/ml). After 48h medium was removed, cells were washed with PBS and then fixed in 4% paraformaldehyde.

After permeabilization with 0.1% Triton X-100, cells were first blocked with 1% BSA (in PBS) for 1h and then incubated with the primary antibodies anti-TREM2 (1:500; goat; Abcam-ab95470) and anti-Iba1(1:500; rabbit; Wako; 019-19741) (in the same 1% BSA solution) and incubated overnight at 4°C. The next day after washing with PBS, cells were incubated with AlexaFluor 488 anti-rabbit and AlexaFluor 555 anti-goat

(both 1:500) , 1h RT. Cells were subsequently washed with PBS three times and stained with DAPI (10 min). Coverslips were mounted on glass slides and observed using a Axiophot fluorescence microscope(Zeiss, Milano, Italy). Images were handled with ImageJ software (NIH).

8. WESTERN BLOTTING ANALYSIS(WB)

Western blot analysis was used to determine TREM2 protein levels both in brain extracts(1 and 6 months), and cell extracts.

Loading Buffer was added to a solution containing 40µg of protein per sample for the brains. Twenty µg were loaded for the cell extracts. Proteins were denaturated at 100°C for 10 min. Samples were electrophoresed on 12% acrilammide gels in MES running buffer(50 mM MES (2-[N-morpholino]ethanesulfonic acid), 50 mM Tris base,1 mM EDTA, 0.1% SDS; ph 7.3) and transferred to a nitrocellulose membrane (0.45nm pore size) with the semi-dry transfer (Biorad).

After 1h of blocking in 5% milk in TBS-Tween 0.05%, membranes were exposed to anti-TREM2(1:500; rabbit; Wako) over night 4°C. After washing three times for 10min with TBST, the membrane was incubated with an anti-goat-HRP secondary antibody (1:3000, Santa Cruz) for 1h, RT. Tubulin (Sigma) or GAPDH (Fitzgerald) were either evaluated as the housekeeping genes to normalize protein levels.

9. RT-PCR

RT PCR was performed to assess the mRNA levels in microglial primary cultures treated with mAb αD11 versus the control (both anti-V5 or blank).

RNA EXTRACTION

RNA from cells treated with mAb αD11, anti- V5 and control was extracted with the NucleoSpin RNA commercial kit (Machery-Nagel):

- ✓ Lysing cells: 350 µL Buffer RA1 and 3.5 µL ß-mercaptoethanol (ß-ME) was added to the cell pellet and vortexed vigorously
- ✓ Filtrating lysate: Viscosity was reduced and the lysate was cleared by filtration through NucleoSpin® Filter (violet ring): Place NucleoSpin® Filter in a Collection Tube (2 mL), applying the mixture, and centrifuging for 1 min at 11,000 x g.

- ✓ Adjusting RNA binding conditions: the NucleoSpin® Filter was discarded and 350 µL ethanol (70 %) was added to the homogenized lysate and mixed by pipetting up and down (5 times).
- ✓ Binding RNA: For each preparation one NucleoSpin® RNA Column (light blue ring) was placed in a Collection Tube. Lysate was pipette up and down 2–3 times and loaded the lysate to the column then centrifuged for 30 s at 11,000 x g. The column was placed in a new Collection Tube (2 mL).
- ✓ Desalting silica membrane: 350 µL MDB (Membrane Desalting Buffer) was added and centrifuged at 11,000 x g for 1 min to dry the membrane.
- ✓ Digesting DNA: DNase reaction mixture was prepared in a sterile 1.5 mL microcentrifuge tube: For each isolation, 10 µL reconstituted was added to 90 µL Reaction Buffer for rDNase. Mixing by flicking the tube. 95 µL DNase reaction mixture was applied directly onto the center of the silica membrane of the column and incubate at room temperature for 15 min
- ✓ Washing and drying silica membrane: 1st wash: 200 µL Buffer RAW2 was added to the NucleoSpin® RNA Column then centrifuged for 30 s at 11,000 x g. The column was placed into a new Collection Tube (2 mL); 2nd wash: 600 µL Buffer RA3 was added to the NucleoSpin® RNA Column and then centrifuge for 30 s at 11,000 x g. Flowthrough was discarded and the column placed back into the Collection Tube; 3rd wash: 250 µL Buffer RA3 was added to the NucleoSpin® RNA Column and then centrifuge for 2 min at 11,000 x g to dry the membrane completely. The column was placed into a nucleasefree Collection Tube (1.5 mL).
- ✓ Eluting RNA: the RNA was eluted in 60 µL RNase-free H2O, and centrifuged at 11,000 x g. for 1 min.

RETRO TRANSCRIPTION

For the reverse transcriptase reaction we used the RT-RTCK-03 kit by EUROGENTEC. The protocol as follows:

- ✓ All required reagents necessary for the RT step were thawed completely and placed on ice, except for the EUroScript, which was kept in the freezer until required use. All reagents were mixed well by inversion and spinned down prior to pipetting.

- ✓ Prepare the RT Reaction Mix

Component	Volume(µl)	Final concentration
10x reaction buffer	1	1x
25mM $MgCl_2$	2	5mM
2.5 mM dNTP	2	500µM each dNTP
Random Nonamers	0.5	2.5µM
RNAse Inhibitor	0.2	0.4 U/µl
EuroScript RT	0.25	1.25U/µl
RNase free water	3.05	
Template	1	10 pg- 200 ng total RNA
Total mix	10	

- ✓ To correct for dispensing losses an excess of reaction mix was prepared. All components were added together, except for the template and mixed thoroughly by inversion. Spinned down.
- ✓ Added the reaction mix to the reaction vial (reaction set up was done on ice)
- ✓ Added the template to individual reactions gently mix by inversion. Spinned down. A negative control containing no RNA template was included.
- ✓ The thermo cycler was programmed with the following parameters: initial step : 10 min at 25°, Reverse Transcriptase step: 30 min 48°C; Inactivation of the RT enzyme: 5 min 95°C.

qPCR

For the the qPCR step according to the kit SsoAvanced Universal SYBR Green Supermix(172-5272)(BIO-RAD), the protocol goes as follows:

- SsoAdvanced universal SYBR Green supermix and other frozen reaction components were thawed to room temperature and mixed thoroughly. They were centrifuged briefly to collect solution at the bottom of tubes, and then stored on ice protected from light.
- Enough reaction setup was prepared for all qPCR reaction by adding all reaction components except the template
- Reaction Setup:

Component	Volume(µl)	Final Cncentration

Forward Primer	1 µl	250nM
Reverse Primer	1 µl	250nM
SsoAdvanced universal SYBR Green supermix (2x)	10 µl	1x
Template	4 µl	100 ng
RNase free water	4 µl	-
Total mix	20 µl	-

- The assay master was mixed thoroughly to ensure homogeneity and equal aliquots were dispensed into each well of the PCR plate
- Samples were added to the wells containing the master mix, the well was sealed with optical film and vortexed 30 seconds or more to ensure thorough mixing of the reaction. Any air bubbles were removed by spinning.
- The plate was loaded onto the real-time PCR instrument and the PCR run was started(Default setting were used to program the thermal cycling protocol).

LIST OF PRIMERS:

PRIMER	SEQUENCE	TM
TREM2 FORWARD	5'- TAT GAC GCC TTG AAG CAC TG-3'	55.2°C
TREM2 REVERSE	3'- AGA GTG ATG GTG ACG GTT CC-3'	56.8°C
GAPDH FORWARD	5'- GTG TTC CTA CCC CCA ATG TG-3'	55.6°C
GAPDH REVERSE	3'- AGG AGA CAA CCT GGT CCT CA -3'	57.6°C

10. FACS

FAC analysis was used to determine the efficiency of the isolation of microglia from adult brain protocol. After isolation cells were resuspended in PBS and analyzed by flow cytometry. Data analysis was performed with FlowJo (http://www.flowjo.com/) software.

11. STATISTICAL ANALYSIS

Statistical analysis was conduct using the Microsoft Excel Analysis Toolpak (http://www.excel -easy.com/data-analysis/analysis-toolpak.html). Normality of the parameters for the morphological evaluation was evaluated assessing their distribution

function statistics, skewness and kurtosis(skewness of the parameters is significant if SKEW> 2*SQRT(6/COUNTS)). ANOVA and T-tests were performed through the same Microsoft Excel toolkit. Analysis of RT-qPCR data was performed with the $\Delta\Delta C_T$ method using using the non-parametric Mann-Whitney Test on Origin software (http://www.originlab.com/). For all tests, α was set to 0.05.

RESULTS

1 CHARACTERIZATION OF MICROGLIA IN AD11

1.1 MORPHOLOGIC ANALYSIS

As a first step, I went to evaluate morphologic parameters on animals of one month of age to detect any changes in the earliest pre-symptomatic phase, where immune changes are in AD11mice are already detected (see AD11 mice introduction). Three to six animals per group were used (WT-, VH-, AD11 mice), both enthorinal cortex and hippocampus were evaluated.

The results of the immunohistochemistry in **Fig.1.1.1** show a significant decrease in branching length per cell, branching complexity and branching density of the microglia in AD11 enthorinal cortex compared to controls , indicating that at this early stage of the neurodegeneration thereis already a decrease of the microglial branching. Thus a subpathological impairment can be already observed at this age. VH mice seem to show a more subtle decrease in branching complexity that their AD11 counterparts, while also in the Branching length per cell parameters we see a significant, though barely, increase.

The result in **Fig 1.1.2** for the hippocampus show less pathological changes than in the enthorinal cortex, since only a decrease in branching density and complexity but not branching length per cell can be observed. Interestingly, there is also a slightly significant decrease in cell density in AD11 with respect to controls, as yet another sign of microglia subtle impairment. In many of the parameters VH show an intermediate phenotype between full on AD11 and WT with decreased branching density and complexity.

At 6 months of age, we found the same tendency towards a decreased branching in the enthorinal cortex (**Fig. 1.1.3**), where AD11 mice demonstrate a reduced branching length per cell and branching density. The same result can be observed in the hippocampus(**Fig. 1.1.4**) where branching length per cell, branching complexity and branching density are all decreased compared to the WT or VH. Yet no difference in cell density was detected. At 6 month there is barely a difference between VH and WT.

The microglial impairment evaluated through morphological parameters is therefore present both at 1 and 6 months of age.

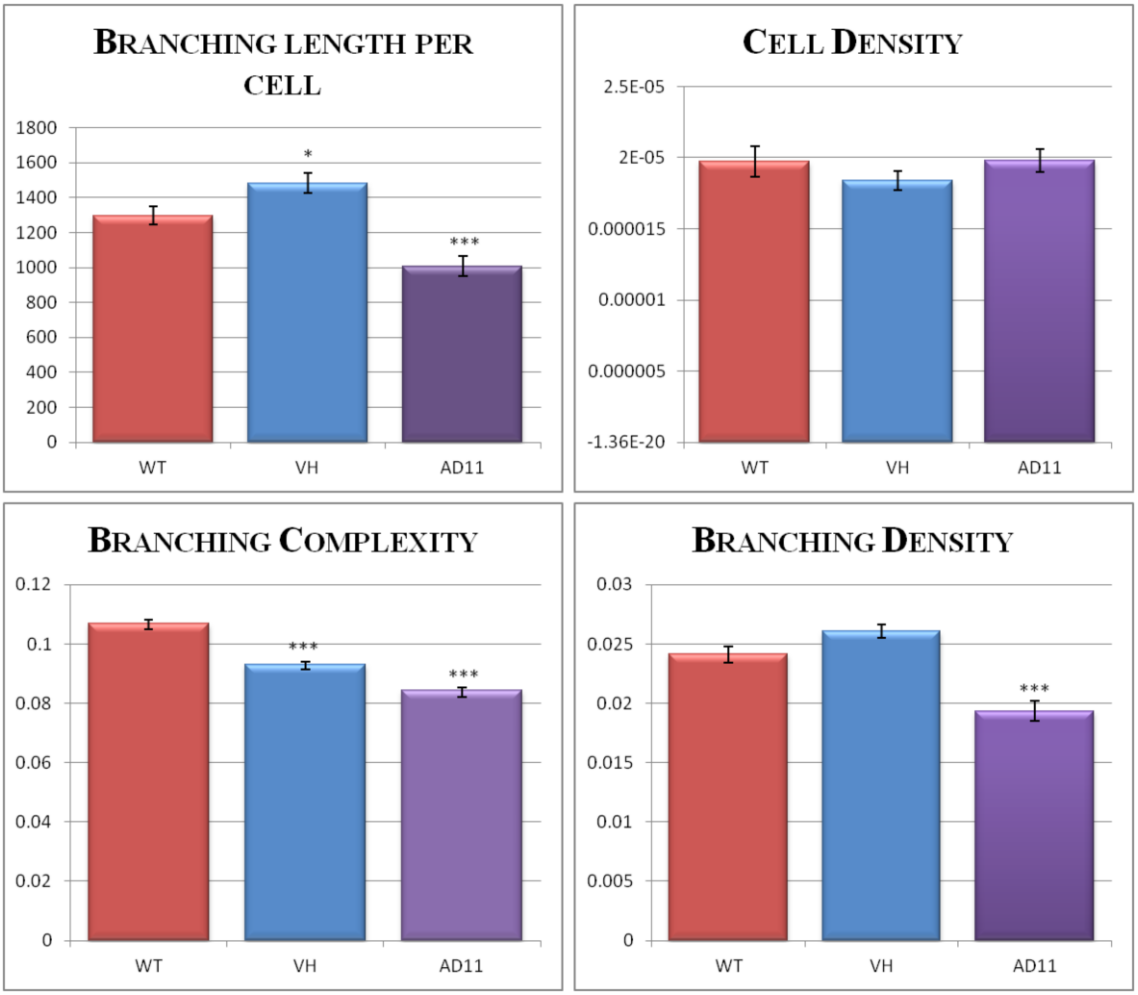

Figure 1.1.1: These histograms show values on 1 month old animals (enthorinal cortex). Bars are representative of the mean ± s.e.m.

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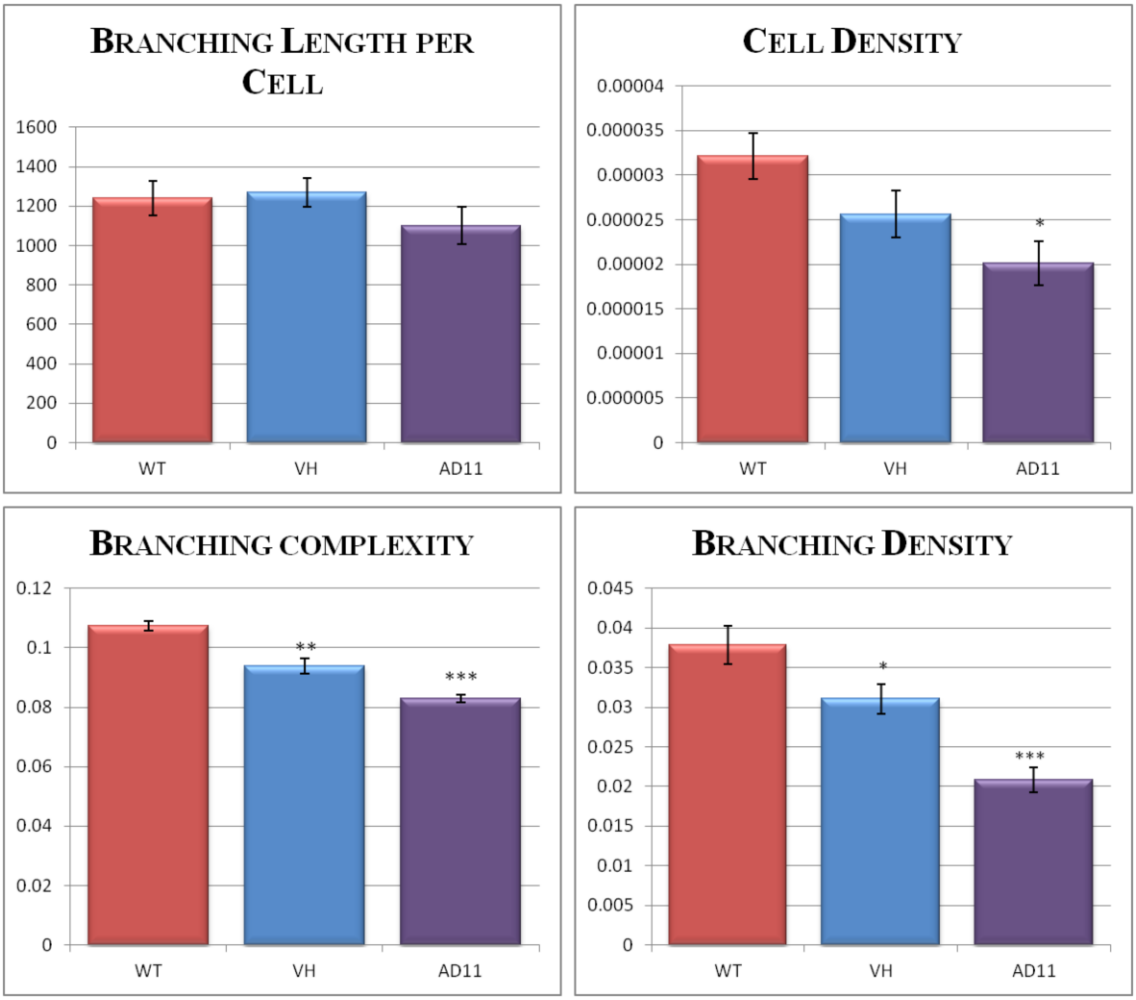


Figure 1.1.2: These histograms show values on 1 month old animals (hippocampus). Bars are representative of the mean ± s.e.m.

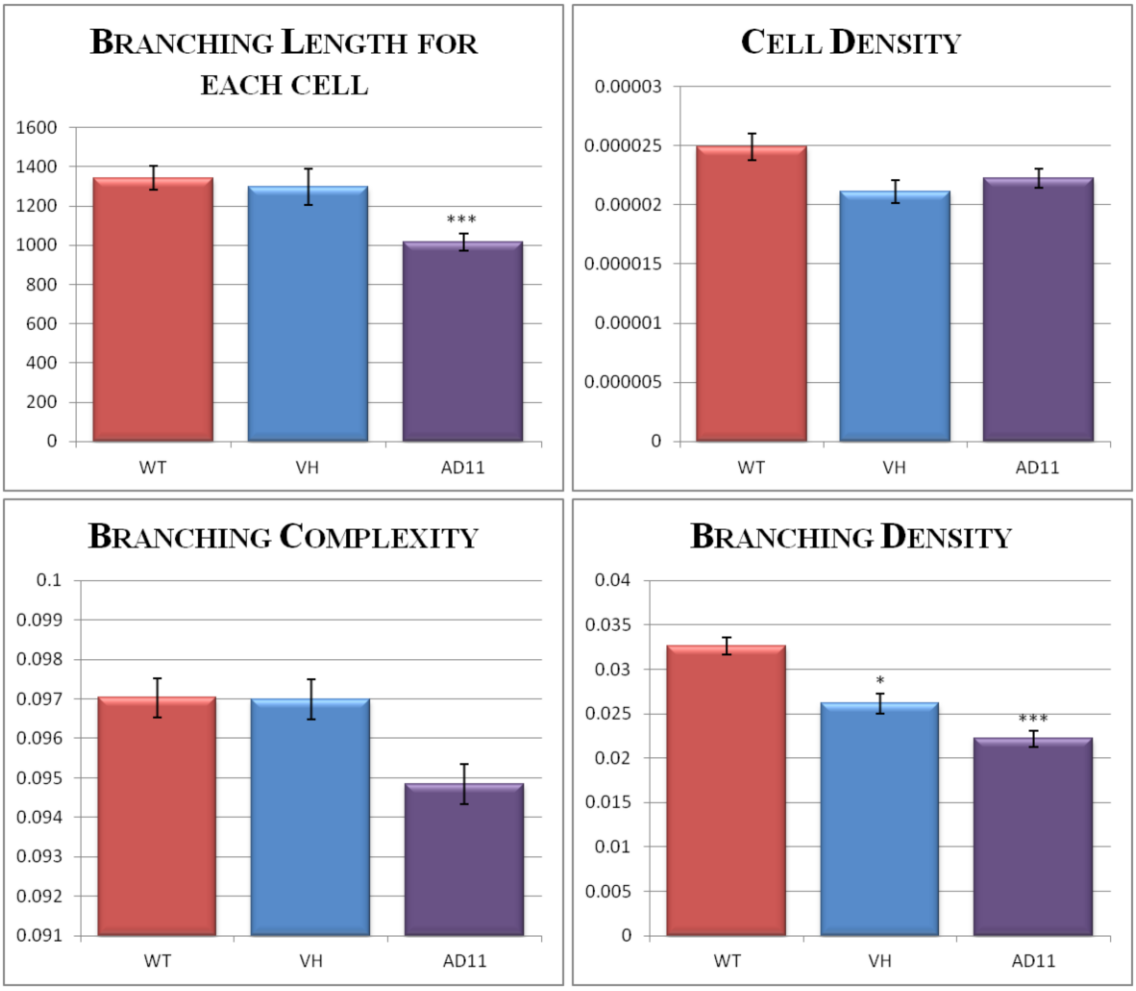


Figure 1.1.3: These histograms show values on 6 month old animals (enthorinal cortex). Bars are representative of the mean ± s.e.m.

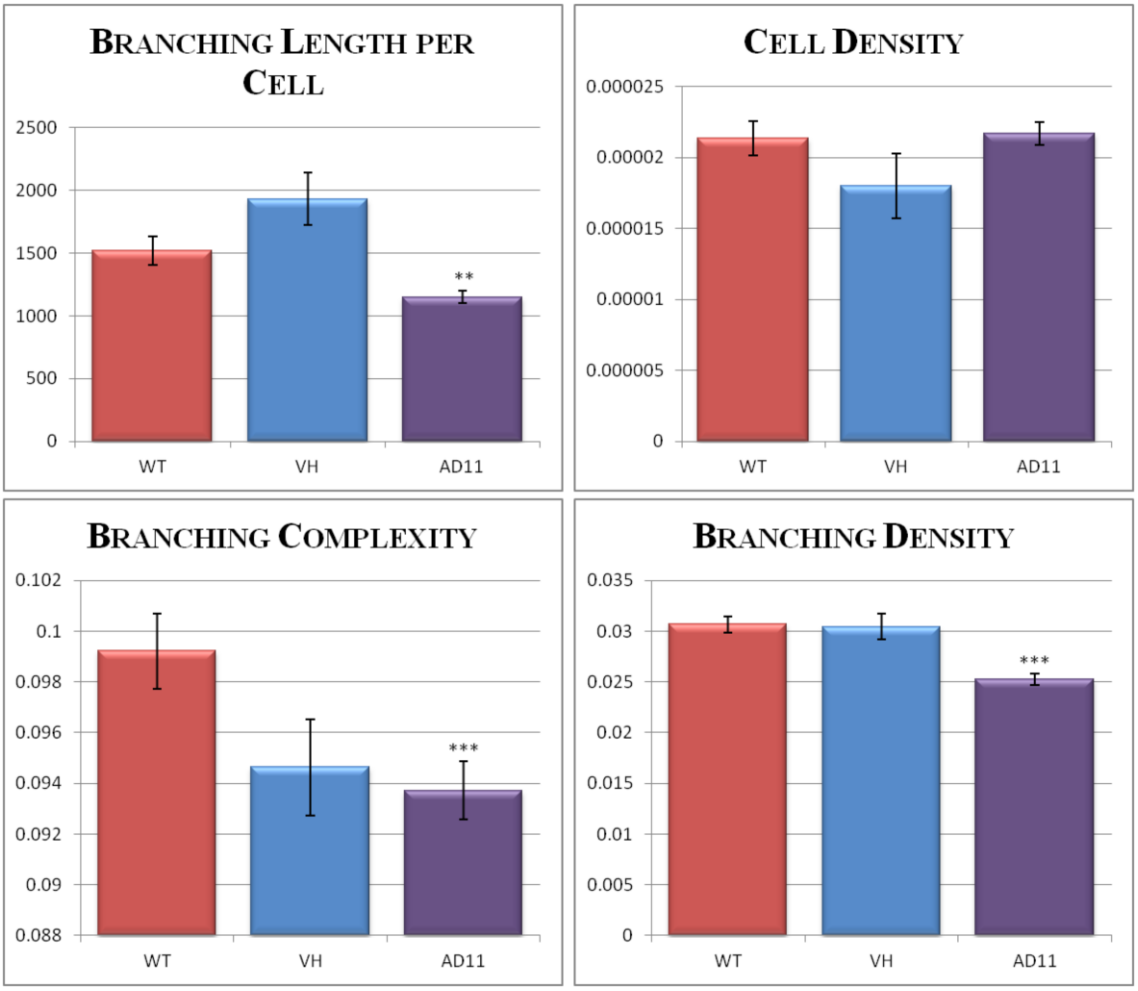


Figure 1.1.4: These histograms show values on 6 month old animals(hippocampus). Bars are representative of the mean ± s.e.m.

1.2 Western Blotting Analysis of a protein of interest: TREM2

Since the morphological analysis revealed subtle changes that could not be associated with neuroinflammatory/proinflammatory phenotype of these myeloid cells but that could at best contribute to a decreased surveillance ability due to a diminished branching density, the next step in the project consisted in assessing the levels of the TREM2, a protein that can be considered a marker of phagocytic activity of microglia (Rivest 2015) and strongly related to AD neurodegeneration (Karch, Cruchaga et al. 2014).

TREM2 protein levels were assessed by western blotting of extracts of tissue samples from 1 and 6 months old mice (**Fig. 1.2.1**)(N=4 for each age and genotype).

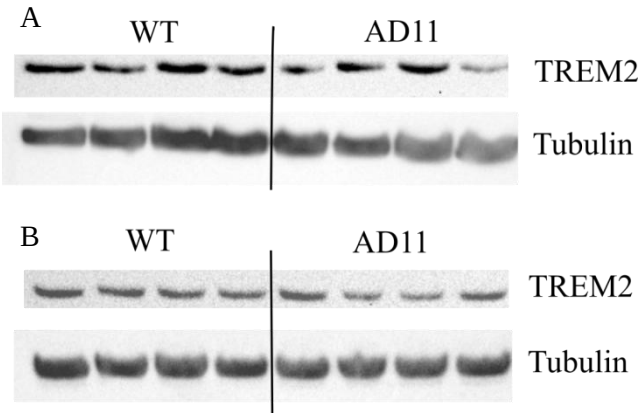
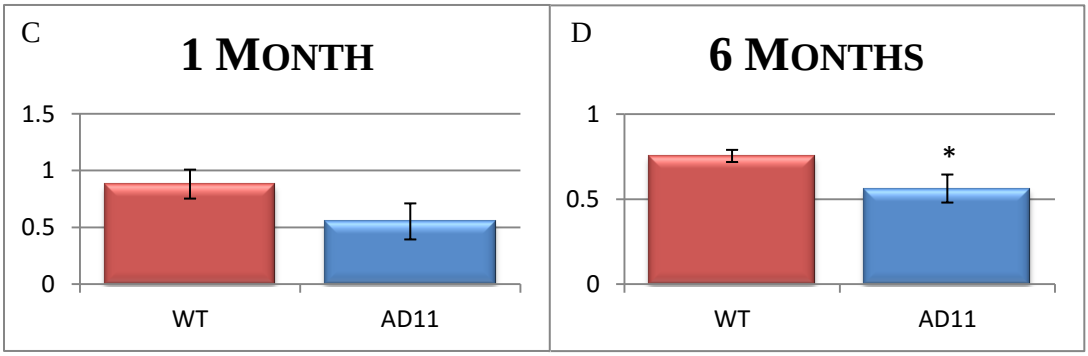


Figure 1.2.1: WB of tissue samples from (A) 1 month old mice, (B) 6 months old and quantification of (C) 1 and (D) 6 months relative levels of TREM2. Bars are representative of the mean ± s.e.m.



WB analysis revealed a decrease in TREM2 protein levels, that at 1 month is a tendency since the p value is 0.07, while it reached statistical significance at 6 months of age.

2 MIMICKING IN VITRO OF NGF DEPRIVATION: FOCUS ON TREM2

To confirm the effect of NGF deprivation on microglia, an *in vitro* approach was used. Thus, I investigated directly on microglia primary cultures whether the treatment with the neutralizing antibody mAb αD11 could lead to the same modulation of the expression of TREM2 observed in AD11 mice.

2.1 IMMUNOCYTOCHEMISTRY AND WESTERN BLOT FOR TREM2

The first step in acquiring that knowledge was performing a simple ICC to assess the localization of the TREM2 protein (Fig 2.1). We found that TREM2 is expressed on the cell surface and in nuclei of IBA-1 immunoreactive cells, i.e. on microglia.

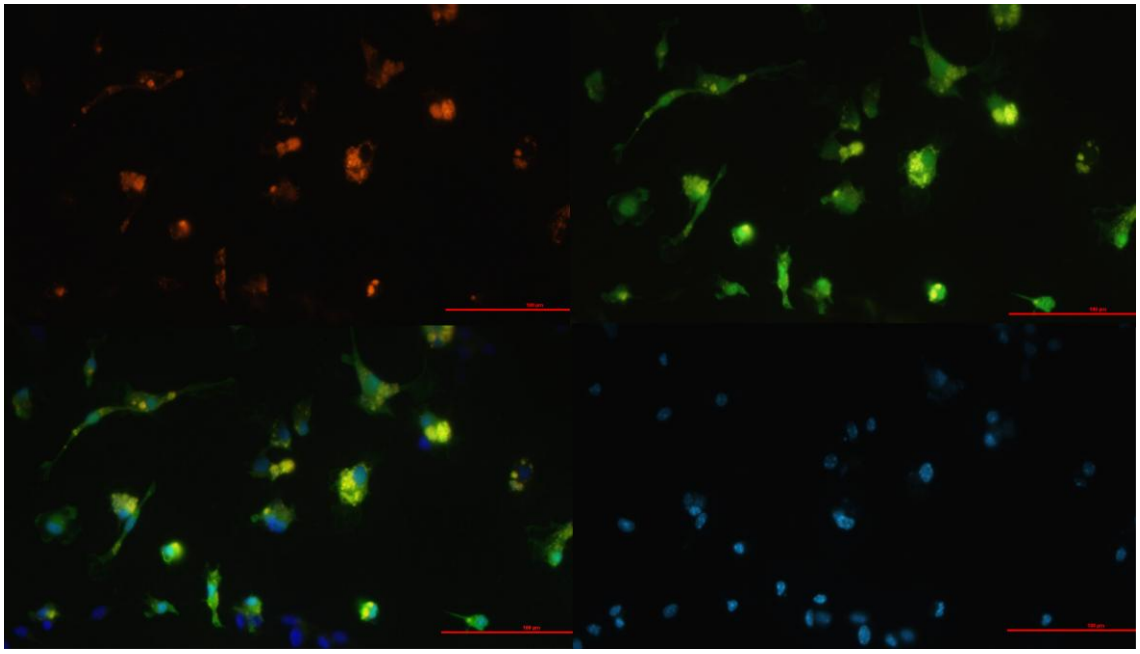


Figure 2.1.1: ICC for TREM2(red), Iba1(green), DAPI (blu) on primary culture of microglia.

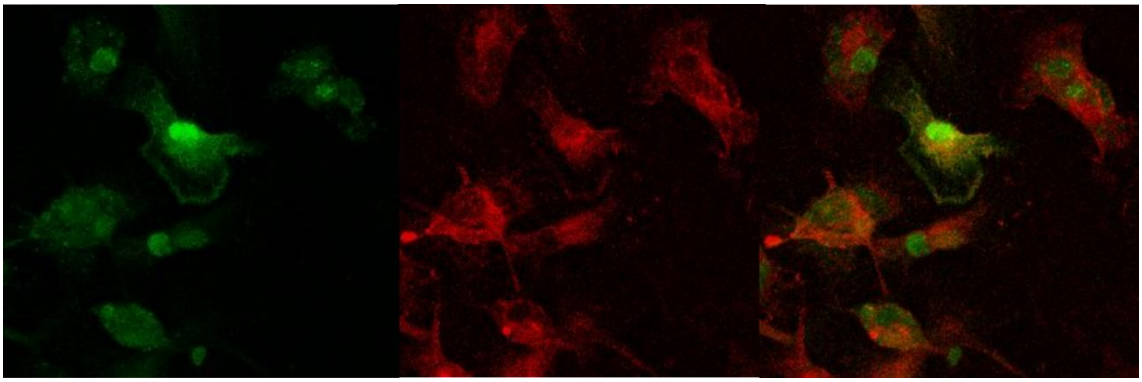


Figure 2.1.2: Zoom in on the staining of TREM2(red) and Iba1(green) on a ICC on microglia.

2.2 RT-qPCR

To specifically analyze the effects of NGF deprivation on TREM2 expression, we performed RT-qPCR to quantify mRNA expression after applying the usual treatment with mAb αD11 on primary microglia. Treatment of cells was done according to the protocol in MATERIAL AND METHODS - 5 TREATMENTS WITH MAB αD11 FOR RT-PCR AND WB.

RT-qPCR reveals that indeed the neutralization of NGF by mAb αD11 induces a downregulation of the expression of TREM2 in vitro, while the treatment with the anti-V5 antibody does not change the expression of TREM2 mRNA. Thus we were able to replicate in vitro the observation performed on AD11 mouse tissues and we demonstrated that to the effect is specific for the αD11 antibody and it is thus a consequence of NGF deprivation.

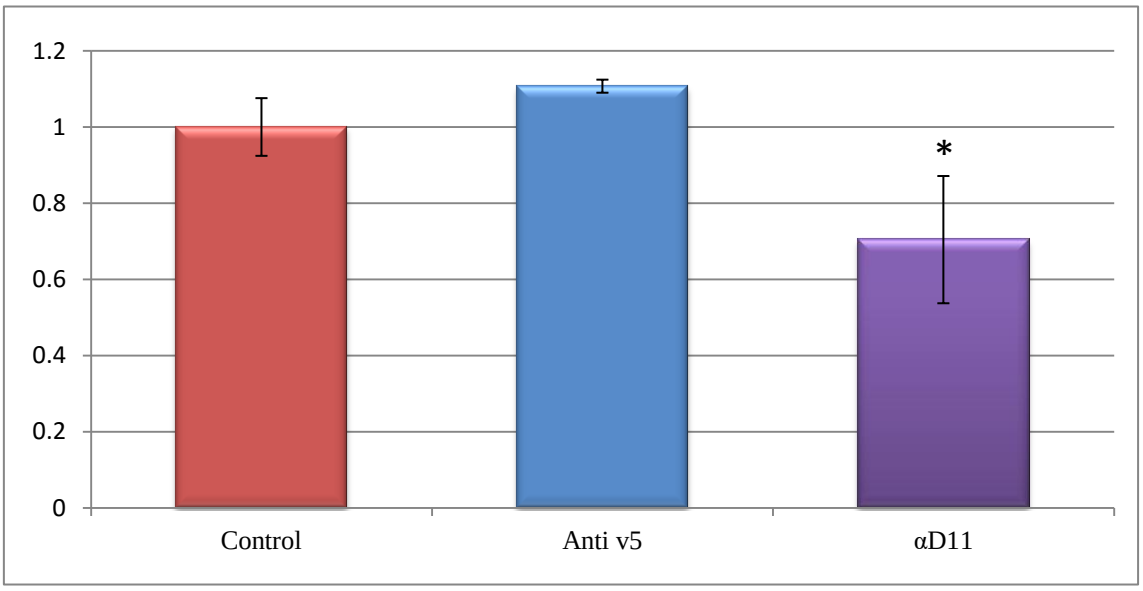


Figure 2.3.1: The histogram shows RT-qPCR results. A Mann-Whitney test was performed with the Origin software. Bars are representative of the mean± s.e.m.

2.3 WB

To confirm the effect of NGF deprivation on TREM2 on a protein level we performed WB for extracts from BV-2 microglial cells treated for 48h with the antibody (plus a blank and the control antibody anti-V5) as for protocol (MATERIAL AND METHODS – 5 TREATMENTS WITH αD11 FOR RT-PCR AND WB).

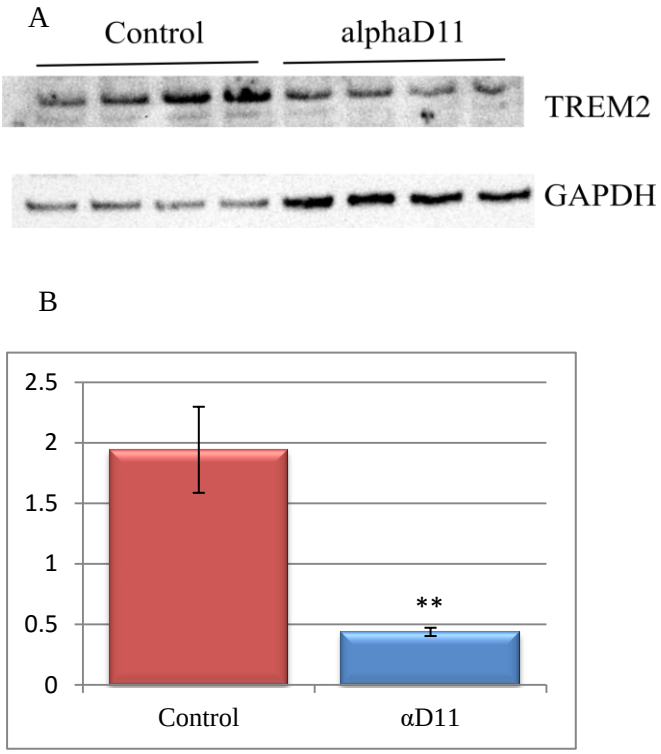


Figure 2.2.1: WB of cell extracts from (A) Control versus αD11 and quantification (B) of the relative levels of TREM2. Bars are representative of the mean ± s.e.m.

As we can see in **Fig. 2.2.1**, cells treated with αD11 show decreased levels of TREM2 compared to controls (B), a result that is thus consistent with RT-qPCR analysis.

Thus both *in vitro* and *in vivo* experiments point toward a regulation of TREM2 expression by NGF that can be transposed in a relationship between NGF and microglial phagocytic power. Moreover, a diminished phagocytic power and in general microglial impairment correlates with an increase in Aβ accumulation and thus a decrease in TREM2 expression in AD11 mice directly correlates with the onset of the AD-like pathology.

DISCUSSION

The phenotype of AD11 anti-NGF mice triggers big scientific questions and unknowns, contrary to other AD mouse models in which there is an over-expression of proteins that are endpoints of the neurodegeneration, it is due to the neutralization of a peptide, NGF, that is upstream to these endpoints.

Indeed, the most common AD models use the ectopic overexpression of proteins associated with human AD (i.e. APP, PS1, tau) in order to induce a phenotype that resemble that of the human pathology. The proteostasis (protein homeostasis) is thus led into overdrive causing the development of clinical and pathological features of AD although in varying degrees and different combination. One key problem though is that existing mouse models do not express all of the AD phenotypes: no "perfect" model exists (Hall and Roberson 2012) and often these can be used only to dissect certain parts of pathological process such as Aβ aggregation.

The AD11 mouse stands out as a model that is able to present AD-like features without interfering with the proteostasis mechanisms (at least not in known ways) thus representing a door into some hidden mechanism behind AD. Which mechanism affected by NGF deprivation is the primary seed that leads to neurodegeneration is unknown, since NGF biological action is affecting multiple systems in the organism (Levi-Montalcini, Science 1986).

My thesis research was a first approach in analyzing the involvement of microglia in the induction of the AD phenotype in AD11 mice. This meant specifically looking for an interaction between microglia-NGF deprivation first by assessing microglial status in AD11 mice and then by recreating in vitro the same environment for microglia by treating cells with the anti-NGF antibody expressed in AD11, mAb αD11.

Since in AD11 mice immune changes are already significant in the preclinical phase of the AD-like pathology (D'Onofrio et al., 2011), namely already at 1 month of age until 6 months (i.e. until an age in which the accumulation of plaques starts and behavioral deficits appear), the 1-to-6-months time span was deemed appropriate to evaluate a specific microglial role in inducing the AD-like phenotype.

The difficulty in this work is related to the fact that there is no one way to characterize microglia phenotipically. Approaches have consisted in determining cells soma size, processes total length per cell, and a bunch of other morphological parameters that have the supposed role of representing activation. Thus, the first step in analyzing these cells into AD11 brains was to look for subtle morphological changes in two areas which are the most affected by neurodegeneration in Alzheimer's Disease, the hippocampus and the enthorinal cortex.

My personal selection of morphological parameters focuses on branching since it is through their processes that microglia exert their biological action such as macropinocytosis or phagocytosis (and clearance of Aβ depends on these mechanisms). These parameters were: (a) the BRANCHING LENGTH PER CELL which is a measure of the total length of the processes for one single cell, a parameter that has already been associated with activation before (Torres-Platas, Comeau et al. 2014). Indeed, microglial activation has been linked to processes retraction and acquisition of an "ameboid" morphology so that would in this case entail a decrease in BRANCHING LENGTH PER CELL. Another parameter is the BRANCHING COMPLEXITY which answers to the question "how ramified are the processes?". A third morphological parameter is the BRANCHING DENSITY**.** This particular parameter was calculated by dividing the sum of the branching length of every cell in a slice and the volume of said slice to represent some sort of *density* of branching. This value should be correlated to the ability of microglial processes to scour the extracellular environment. A decrease of branching density would then reflect a diminished clearance ability and a sign of microglial impairment. I decided to make the process length a function of volume because I supposed it would have been more specific and sensitive to pre- symptomatic phase subtle changes as opposed to the more common branching length per cell which is most likely linked to downright activation. Finally, we calculated the CELL DENSITY. This parameter is pretty straight forward and corresponds to cell count/stack volume. The aim of the parameter is to assess microglial proliferation/cell death. As a first step, we went to evaluate morphologic parameters on animals of one month of age to detect any changes in the earliest pre-symptomatic phase, where immune changes are in AD11 mice are already detected (see AD11 introduction).

The analysis was performed using immunohistochemistry to detect a specific microglia marker, Iba-1. This technique enables to observe in a very precise manner microglia and

microglial processes and it is the to-go experimental approach when asking about morphology. Morphological evaluation of microglia showed that in AD11 mice there are indeed some changes during the pre-symptomatic stage (RESULTS - 1.1 MORPHOLOGIC ANALYSIS). Compared to WT, in AD11 mice microglia shows a decrease in many morphological parameters assessed in this study. As previously mentioned, a decrease in branching density correlates historically to activation since retraction of processes is one of the steps toward the ameboid activated microglia. However, these changes, are a sign not of overt classical "activation" in AD11 mice since we did not observed any "ameboid cells". Thus, this decrease in branching density (as a measure of surveillance power) might underlying subtle sub-clinical impairment in the homeostatic functions of the microglia. In favor of impairment versus classical activation there the fact there in AD11 mouse brains, contrary to other mouse models of AD (Kamphuis, Orre et al. 2012, Gomez-Nicola, Fransen et al. 2013), no apparent microglial proliferation could be observed. Interestingly enough, phenotypic changes but not proliferation underlies microglial response in patients of Alzheimer's Disease, thus placing AD11 closer to the human phenotype than other AD mouse models (Serrano-Pozo, Gomez-Isla et al. 2013, Smith and Dragunow 2014).

Since a microglial impairment such as the one demonstrated in the aging brain might lead to a decreased phagocytic ability of the microglia, during the second part of my thesis project I started to focus on TREM2.

This protein has been correlated with the phagocytosis of Aβ (Rivest 2015) especially in non inflammatory conditions (Takahashi, Rochford et al. 2005) and it has been associated with AD as a very important risk factor (Karch, Cruchaga et al. 2014).

However, controversial results were obtained in mouse models that were analyzed to understand the relation between TREM2 expression and Aβ deposition. On one hand, Jay et al. recently found that levels of TREM2 expression are up-regulated in myeloid cells that accumulate around Aβ plaques in two mouse models of Alzheimer's disease, similarly to human AD (Jay, Miller et al. 2015). The same authors found that when APP mice were crossed to Trem2-deficient mice, the number of myeloid cells decreases resulting in reduced brain inflammation and astrocytosis, fewer Aβ plaques in the hippocampus, and attenuated hyperphosphorylation of the tau protein, as compared with APP/PS1 mice with intact Trem2.

On the other hand, another study showed that TREM2-deficient 5XFAD mice exhibited higher hippocampal amyloid burden and neuronal loss than 5XFAD animals in which TREM2 was normally expressed (Wang, Cella et al. 2015). These mice also showed a significant increase of the insoluble form of Aβ40 and Aβ42 in the hippocampus. In the study, TREM2 appeared also to be important for microglial survival since, TREM2-deficient mice had reduced number of microglia, particularly in the areas surrounding senile plaques. The decrease number of microglia cells could be explained by the fact that this microglia was shown to undergo apoptosis. Thus, TREM2-deficient microglia might be incapable of sustaining reactive microgliosis and undergo apoptosis rather than becoming activated and expanding. This may be the critical mechanism underlying the failure of TREM2-deficient microglia to clear Aβ and explains the amyloid burden in this mouse model of AD. Also supporting this conclusion are the results of a study by Hickman and colleagues (Hickman, Kingery et al. 2013), showing that TREM2 is highly expressed by resident microglia in wild-type mice. Moreover, Kleinberger et al. (Kleinberger, Yamanishi et al. 2014) found that the part of TREM2 that extrudes from the cell surface is cleaved by a protease and released in the extracellular space. The same authors showed that that soluble TREM2 levels are decreased in the cerebrospinal fluid of patients with Alzheimer's disease and frontotemporal dementia. These findings further suggest that compromised TREM2 function increases the risk of these two forms of dementia.

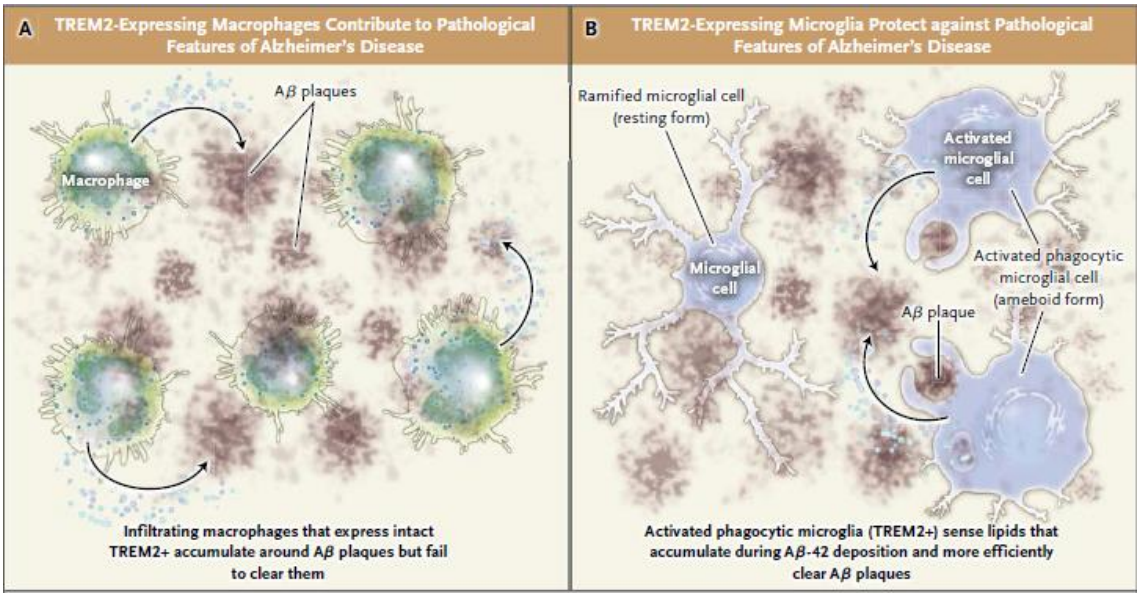


Figure 1. Different Takes on TREM2(Tanzi 2015).

Thus, the results obtained so far in AD mice are very contradictory but these contradictions probably arise from the fact that these mice are produced in a certain way, they develop AD because of ectopic expression of a human protein and not because of some endogenous mechanism. Disruption of microglia is thus doomed to be fairly different depending on the aftermath of the genetic perturbation. Moreover both these papers are lacking one of the most critical piece of data, behavior analyses of the cognitive decline and/or improvement.

In our study, whole brain WB analysis revealed that there is actually a decrease in TREM2 protein levels in AD11 brains compared to WT. This decrease in an environment deprived of NGF has been replicated in vitro using the αD11 antibody on microglial cultures. In vitro, TREM2 levels were also analyzed by RT-qPCR assessing mRNA levels in response to treatment with αD11. The analysis is consistent with WBs.

NGF deprivation in AD11 thus causes a microglial impairment already detectable at 1 month. It is a disruption of the homeostatic functions of the microglia that underlies a deficit in the phagocytosis of the Aβ peptide by microglia, a deficit revealed by a decrease in TREM2 levels.

FUTURE AND PERSPECTIVES

First and foremost, I think adding additional time points at 8 and 12 months is required, since later stages in the pathological processes have to be assessed. It would complete the picture of the microglial involvement in the induction of the AD11 phenotype from the morphological point of view.

Moreover, since the WB results from all brain samples are far from being definitive, first of all I intend to increase the number of animals to help with the statistics. Second of all in our lab we are setting up a protocol to isolate microglia from the adult brain (MATERIAL AND METHODS 4 MICROGLIA ISOLATION FROM ADULT MOUSE BRAIN).

To this aim we will rely on CX3CR1-GFP+ mice in which GFP expression is restricted to microglia. GFP+ cell sorting is also possible to better isolate the population. AD11 mice were crossed with CX3CR1-GFP+ mice (www.jax.org/strain/005582), to get GFP^{+} microglia. The protocol still has a low yield and there is a lot of debris (**Fig.1** and **2** picture first results) so it will take some time to modify the parameters and get a more clear result.

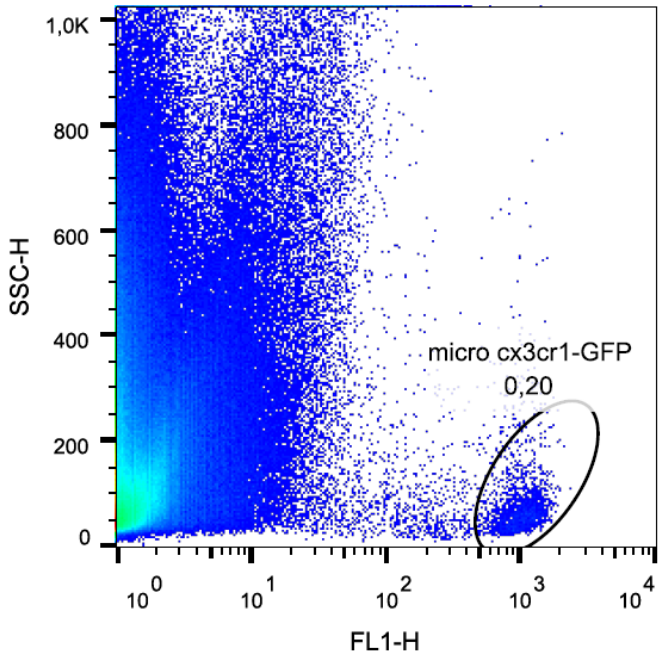


Figure 1: Facs result for a sample obtained with the isolation protocol. On the y-axis we have fluorescence, on the y-axis side scattering (FlowJo).

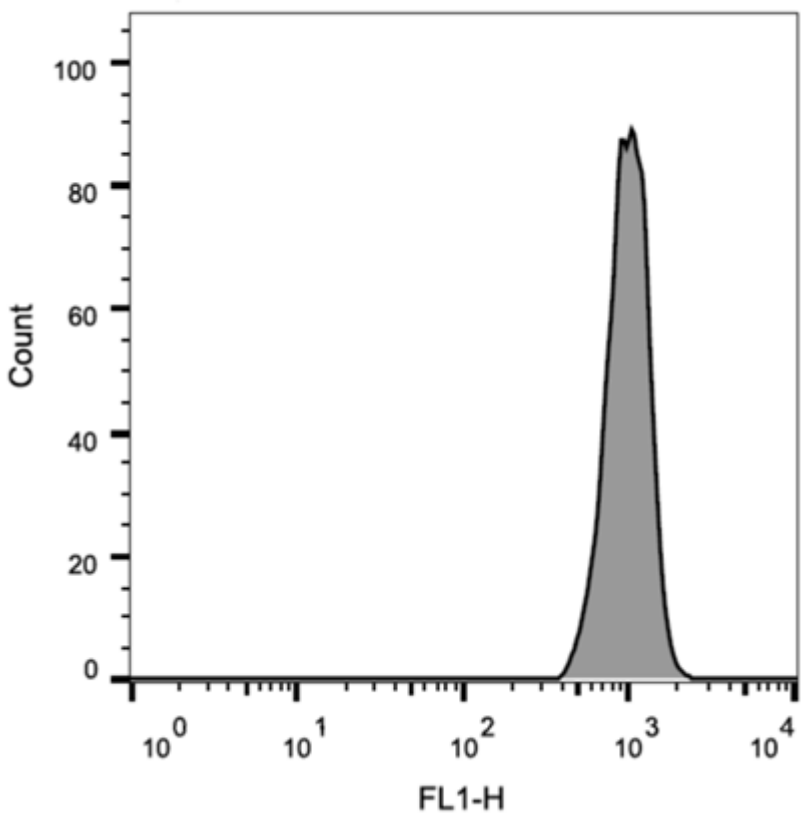


Figure 2: Count histogram that shows clearly the peak of fluorescent microglia (FlowJo).

After this problem is overcome, WB and RT-qPCR will be performed again on a more specific sample. Setting this protocol will also mean being capable of performing functional assays such as phagocytosis assays to determine first hand impairment of microglia isolated from AD11 brains. Macro-arrays or RNAseq techniques might also offer more data to really get into these cells role in AD11 mice.

Finally, it would be interesting to see in vivo microglial behavior in AD11 mice using techniques such as two photon microscopy.

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